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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/36, C12N 15/31, C12P 19/56 — A1

(11) International Publication Number: WO 96/10581

(43) International Publication Date: 11 April 1996 (11.04.96)

(21) International Application Number: PCT/FI95/00537

(22) International Filing Date: 29 September 1995 (29.09.95)

(30) Priority Data:
944556 30 September 1994 (30.09.94) FI

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(81) Designated States: AU, CA, CZ, FI, HU, JP, KR, NO, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROCESS FOR PRODUCING ANTHRACYCLINES AND INTERMEDIATES THEREOF

(57) Abstract

The present invention pertains to a process for producing anthracyclines and intermediates thereof by expressing in a foreign *Streptomyces* host a DNA fragment relating to the biosynthetic pathway of anthracyclines and, if desired, the intermediates obtained are converted to anthracyclines or aglycones thereof using e.g. non-producing *Streptomyces* mutant strains.

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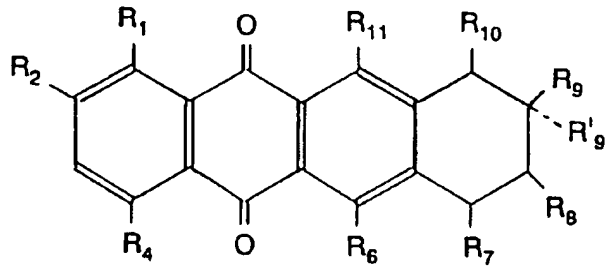
Process for producing anthracyclines and intermediates thereof

The present invention pertains to a process for producing anthracyclines and intermediates thereof by expressing in a foreign host a DNA fragment relating to the biosynthetic pathway of anthracyclines and, if desired, the intermediates obtained are converted to anthracyclines or aglycones thereof using non-producing mutant strains.

Polyketide antibiotics are a broad and variable group of compounds which are composed of poly-β-ketomethylene chain $[CHRO]_{4-20}$. A common feature of polyketides is their biosynthetic route which is similar to the biosynthesis of fatty acids. Katz, L. and Donadio, S. (1993) have recently published a review article concerning polyketides. As their structure the antibiotics of anthracycline group are aromatic polyketides, the common structural body of which is 7,8,9,10-tetrahydro-5,12-naphthacene kinone of the general formula (A)



To this structural body one or more sugars and other substituents are attached. The structural body of the molecule, to which the sugars are attached, is called an aglycone. Anthracyclines are discussed more specifically e.g. in the article of A. Fujiwara and T. Hoshino (1986). Several anthracyclines are cytostatically active and thus they are of continuous interest.

To find new anthracyclines screening of *Streptomyces* bacteria from the soil and mutation thereof are used. To modify known anthracyclines synthetic methods have been used, whereby chemical groups are added to or removed from either the aglycone

or the sugar moiety. Similarly, biotransformation is used, wherein in living cells molecules are modified which have been produced by other production strains or by synthetic methods. Some anthracyclines have also been produced by synthetic methods.

The hybrid antibiotic technology has been disclosed as a new technology in the preparation of new antibiotics. It has been established to comprise production by genetic engineering of molecules which have structural features of natural products of two strains. The process is described in the publication of H.G. Floss: "Hybrid antibiotics – the contribution of the new gene combinations" (1987). The hybrid antibiotic technology gives an opportunity to controlled production of new compounds.

Cloning of actinorhodin genes from *Streptomyces coelicolor* (Hopwood *et al.*, 1985) can be considered as the pioneer work in the molecular biological study of polyketide antibiotics and at the same time of streptomycetes. In 1987 Malpartida *et al.* reported about the hybridization of different polyketide producers to the *act*I and *act*III DNA fragments and thereafter genes of the polyketide synthase (PKS) domain have been identified in many *Streptomyces* species exploiting the homology. Sequencing of these genes has shown that the genes are strongly conserved and include three Open Reading Frames, ORF 1, 2 and 3. The products of these three genes are needed for the formation of the linear polyketide bound to the enzyme complex. For the optimal formation of the correct product encoded by the PKS–genes five ORFs are needed in tetracenomycin (Shen and Hutchinson, 1993). The sequenced aromatic PKSs are given in Table 1.

Table 1. Cloned and sequenced gene domains encoding polyketide synthase of aromatic polyketide antibiotics

Strain	Product	Reference
S. coelicolor	aktinorhodin	Fernandez-Moreno, M.A. *et al.* 1992 Hallam, S.E. *et al.* 1988
S. violaceoruber	granaticine	Sherman, D.H. *et al.* 1989
S. glaucescens	tetracenomycin	Bibb, M.J. *et al.* 1989
S. rimosus	oxitetracycline	Kim, E-S. *et al.* 1994
S. cinnamonensis	monensine	Arrowsmith, T.J. *et al.* 1992
S. griseus	griseusine	Yu, T-W. *et al.* 1994
S. roseofulvus	frenolisine	Bibb, M.J. *et al.* 1994

Polyketide synthase (PKS) is a multienzyme complex which functionally reminds the synthase of long chain fatty acids. The separate components of actinorhodin PKS are so called actORF1 ketoacyl synthase (KS); actORF2 homologous to KS may effect on the length of the polyketide chain (McDaniel, R., *et al.*, 1993); actORF3 acyl carrier protein (ACP); actORF5 ketoreductase (KR) and actORF4 cyclase/dehydrase, which may be responsible for the aromatization of the first ring.

The most part of the biosynthetic anthracyclines are formed via the aklavinone intermediate phase, whereafter the compound is glycosylated or it is modified by adding e.g. hydroxyl or methyl groups. Modifications can occur also after the glycosylation. The biosynthesis of aklavinone and anthracyclines which are further formed therefrom are described e.g. in "Advances in bioconversion of anthracycline antibiotics" (1989) of U. Gräfe *et al.*, and in the references cited therein. The

biosynthetic route of the nogalamycin aglycone being formed of ten acetates is evidently analogous to the biosynthesis of aklavinone. (Figures 1A and 1B).

Description of the invention

A DNA fragment cloned from *Streptomyces nogalater* can be used according to this invention to combine the different phases of the biosynthetic route of anthracyclines, whereby hybrid anthracyclines and precursors of anthracyclines can be produced. This happens by transferring the cloned DNA fragment to a *Streptomyces* strain which produces anthracyclines or, alternatively, to a non–producer of anthracyclines.

The DNA fragment of *Streptomyces nogalater* including in the biosynthesis of anthracycline and being cloned according to this invention caused surprisingly production of anthracycline precursors in *S. lividans*, a host which does not produce anthracyclines. On the basis of the structures of the compounds obtained, the DNA fragment was supposed to include most of the genes needed for the biosynthesis of anthracycline aglycones. By complementation of mutant strains, analyzing the hybrid products and sequencing the DNA fragments we have been able to show that the DNA fragment comprises

- the activity responsible for the election of the starting unit which defines the side chain of the 9–position (*S. galilaeus* hybrid products),
- the polyketide synthase genes,
- the gene of the enzyme which is needed for removing the hydroxyl in 2–position, (ketoreductase),
- the methyl transferase gene needed for the carboxylic acid esterification,
- the mono–oxygenase gene.

This DNA fragment and anthracycline precursors produced by it have further been used to produce hybrid anthracyclines.

The present invention enables one to produce some known cytostatically active anthracyclines (auramycins) as well as prior unknown compounds. Use of the

polyketide synthase of anthracyclines in the production of hybrid anthracyclines has not been described previously, neither the change of the starting unit of polyketide synthesis by transferring genes to a foreign host. Further, there is no prior disclosure of the cloning of genes of the biosynthetic pathway of nogalamycin produced by *S. nogalater*, or use thereof.

The similarity of the biosynthetic genes of polyketide antibiotics disclosed by Malpartida *et al.* (1987) was the starting point to the discovery of the biosynthetic genes of nogalamycin. The total DNA of *S. nogalater* being cleaved by suitable restriction enzymes was hybridized by the Southern–techniques to the *actI* probe, and thus two hybridizing DNA fragments were obtained. In an optimal case a suitable probe shows one DNA fragment. The use of cross hybridization was, however, considered to be possible as a strategy in identifying the biosynthetic genes, because the signals were strong.

The strategy by which the DNA fragment according to the invention was found was the following: A fragment homologous to the *actI* fragment described by Malpartida *et al.* (1987) was isolated from *S. nogalater*. Said homologous fragment and flanking DNA fragments were transferred into a *S. lividans* strain TK24. Altogether about 20 kb (=kilobase, 1000 bases) were transferred in five fragments into a foreign host. Of these an about 12 kb DNA fragment, pSY15, causes the production of nogalamycin intermediates in *S. lividans*. The recombinant strain obtained was cultivated in a nutrient medium used for anthracycline producers and the product was extracted by suitable organic solvents.

DNA fragments according to the invention were transferred into *Streptomyces* strains described hereinafter as well as to *S. galilaeus* mutants H028, JH003, H061, H036 and H039 given in Table 2, and expressed in them. Said DNA fragments can correspondingly be transferred to other mutants mentioned in the table, depending on what kind of products are desired.

Streptomyces lividans 66, strain TK24, restriction–modification–free strain.

Streptomyces galilaeus ATCC 31615, produces aklacinomycin.

Mutants of *Streptomyces galilaeus* ATCC 31615 (cf. Table 2) (Ylihonko *et al.*, 1994).

Table 2. The products of *Streptomyces galilaeus* mutants; abbreviations used: Akn=aklavinone, aglycone moiety of aclacinomycins; Rhn=rhodosamine; dF=deoxyfucose, CinA=Cinerulose A; Rho=rhodinose

Mutant	Product	Description of mutation
H028	No production	Mutation in PKS–domain
JH003	No production	Mutation in PKS–domain
H061	2–OH–Aklanone acid	No removal of 2–OH
H036	Methyl ester of aklanone acid	The fourth ring does not get closed
H039	1)Aklavinone 2)Akn–Rho–Rho	Amino sugar is missing.
H038	Akn–Rhn	Mutation in glycosylation
H026	Akn–Rhn–dF–Rho	Oxidoreductase is missing
H035	Not identified	Mutation in the glycosylation
H054	1)Akn–Rho–dF–CinA 2)Akn–dF–dF–CinA 3)Akn–Rho–dF–Rho 4)Akn–Rho–dF 5)Akn–dF–dF	Amino sugar is missing

When producing the starting product for biotransformation the host used is preferably *S. lividans*, because it does not itself produce coloured or extractable compounds in the growth conditions used.

When producing an aglycone for biotransformation the bacterial strains producing anthracyclines or non–producing mutants thereof are preferably used, most preferably

non-producing mutants of *S. galilaeus* being transformed with plasmid pSY15 (Fig. 3), carrying the above mentioned 12 kb DNA fragment.

When converting the anthracycline precursors obtained using the plasmid pSY15 to anthracyclines or their aglycones, *S. galilaeus* mutants, e.g. strains JH003 or H028, which do not produce aclarubicin are preferably used.

The DNA-constructions according to the present invention can be constructed by ligating suitable DNA fragments from the domain as described to a suitable vector. Such a vector is preferably the high copy number plasmid pIJ486 capable to amplify in several strains of the genus *Streptomyces* (Ward *et al.*, 1986).

To produce anthracyclines and their precursors strains carrying the pSY15 plasmid are grown preferably in growth media for *Streptomyces* bacteria, preferably in E1-medium, to which thiostrepton has been added to maintain the plasmid carrying strains. The strains are grown in conditions which are advantageous to the producing strain, e.g. in a shaker in bottles, or in a fermenter which is stirred and aerated. After a suitable cultivation time, preferably after 2-7 days the products are isolated according to methods described for bacterial metabolites, preferably e.g. extracting with a suitable solvent, e.g. toluene or chloroform. The extracted compounds are purified with suitable methods e.g. by using column chromatography.

Anthracycline precursors are converted to anthracyclines in strains naturally producing anthracyclines, or mutants thereof. Compounds similar to those naturally produced by the strain are thus obtained, having methyl in their 9-position and hydrogen in their 2-position. In biotransformations auramycinone produced by a *S. galilaeus* strain carrying the plasmid pSY15 is most suitably used as the starting compound, or methyl ester of nogalonic acid produced by a strain carrying the same plasmid which naturally does not produce anthracyclines. In biotransformations most preferably non-producing mutants of anthracycline production strains are used, e.g. mutant H028 or JH003. Biotransformation is effected most preferably by cultivating a strain in a suitable liquid production medium, e.g. in E1-medium, and by adding anthracycline precursors in

suitable amounts. After a suitable time, e.g. 6 to 48 hours, most preferably 16 to 32 hours, the anthracyclines so formed are extracted.

The strains used for transformation (cf. also Table 2) are described in the following.

TK24 is a *S. lividans* strain which in the growth conditions used does not produce coloured secondary metabolites. In other growth conditions it produces actinorhodin, which is an antibiotic differing very much from anthracyclines. The strain does not produce any anthracyclines nor their precursors. When characterizing the products of TK24/pSY15 on the basis of NMR-spectrum compound I was obtained as the primary product, which is possibly an intermediate of anthracycline biosynthesis (cf. Scheme I).

H028 is a mutant of *Streptomyces galilaeus* which does not as such produce anthracyclines or their precursors. However, this strain can be used in biotransformations to convert anthracycline precursors to products similar to aclarubicin. When characterizing H028/pSY15 products it was found that this strain produces auramycinone (Compound II), which is an anthracycline aglycone similar to aklavinone, as well as auramycins which are glycosides of auramycinone, e.g. Compound III. When hydrolyzing auramycins auramycinone is obtained, which also shows that the compounds produced are glycosides of auramycinone. Auramycinone is a useful precursor of anthracyclines, when new anthracyclines are produced by biotransformation. Auramycins have been described to be cytostatic anthracyclines having possible use in cancer chemotherapy. The use of H028/pSY15 for the production of these is new.

H061 is a *Streptomyces galilaeus* mutant, which produces 2-OH-aklanone acid. This is evidently due to a mutation which prevents removal of the hydroxyl in 2-position. H061/pSY15 produces aklavinone, auramycinone and their glycosides similar to aclarubicin. According to the result pSY15 complements the mutation of H061 and comprises thus the gene encoding the 2-position dehydroxylase. This is useful in

producing new hybrid compounds when transformed to a strain the products of which naturally have hydroxyl or a methoxy group in 2–position.

On the basis of the results pSY15 is useful in producing precursors of anthracyclines in strains which naturally do not produce anthracyclines, or when producing hybrid anthracyclines in strains which produce anthracyclines, or in mutants thereof. With it the formation of 9–position side chain can be affected so that the strains which provide a two carbon side chain at this position, do produce compounds which have a one carbon side chain at said position. Possible strains producing anthracyclines which can be modified this way are e.g. *S. galilaeus*, *S. peucetius* and *S. purpurascens*. The anthracycline precursors produced this way are useful in producing new anthracyclines by biotransformations. pSY15 can also be transferred to a strain which normally produces compounds which at 2–position have hydroxyl or a methoxy group. Thereby compounds are obtained which have hydrogen at this position. pSY15 enables also one to produce previously described auramycinone and its glycosides by the new method.

In the following the detailed embodiments of the invention are described as examples of isolation of the DNA fragment from *S. nogalater* strain ATCC 27451, production of nogalamycin precursors in *S. lividans* strain TK24, production of auramycinone in the mutant H028 and their modification to anthracyclines in the mutant JH003. In addition, expression of the DNA fragments according to the invention in the mutants of the strain *S. galilaeus* is described, as well as the compounds produced by these strains.

The main products of the strains TK24/pSY15, JH003/pSY15, H028/pSY15 and H061/pSY15 were characterized.

Brief description of drawings

Fig. 1A Anthracyclines produced by *Streptomyces* strains, and identified precursors thereof. (Starting molecule: propionate.) The numbers of *S. galilaeus* mutant strains producing the intermediates are given in parentheses.

Fig. 1B Anthracyclines produced by *Streptomyces* strains having acetate as the starting molecule.

Fig. 2 Restriction map of the 12 kb continuous DNA fragment cloned from *S. nogalater* genome. The figure discloses also the inserts contained in the pSY plasmids obtained. Plasmid pIJ486 has been used in preparing the pSY vectors. On the basis of sequence comparisons the following functions have been obtained for the open reading frames shown in the figure: 1 = ketoacylsynthase–acyltransferase, 2 = Chain Length Controlling Factor (CLF), 3 = acyl transferring protein; A and B = regulatory genes, C = mono–oxygenase, D = methyl transferase, E = ketoreductase.

Fig. 3 Structure of the plasmid pSY15.

Fig. 4 NMR–spectrum of compound I.

Fig. 5 NMR–spectrum of auramycinone.

Fig. 6 NMR–spectrum of auramycinone–rhodosamine–deoxyfucose.

Fig. 7 NMR–spectrum of auramycinone–rhodinose–deoxyfucose.

Materials used

Bacterial strains and plasmids

The strain *Streptomyces nogalater* ATCC 27451 was used as the donor of genes. The *Streptomyces* bacterial strains used in this work as hosts are listed above. The treatments of *S. nogalater* DNA were effected in the *E. coli* strain XL1-Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac*, [F'*pro*AB, *lac*IZΔM15, Tn*10* (tet^r)] (Stratagene Cloning Systems, California). *E. coli* strains GM2163 (*E. coli* Genetic Stock Center, Department of Biology 255 OML, Yale University, New Haven, USA) and LE392 (Promega) were used in preparing the gene bank and in amplifying the phage DNA.

In *E. coli* the plasmids pUC18/pUC19 (Pharmacia Biotech) were used, and in *Streptomyces* strains the plasmid pIJ486 was used (Ward *et al.*, 1987; obtained from Prof. Hopwood, John Innes Centre, UK).

Nutrient media and solutions used

TRYPTONE-SOYA BROTH (TSB)

Per litre: Oxoid Tryptone Soya Broth powder 30 g.

YEME (Hopwood *et al.*, 1985., p. 239)

Per litre: Yeast extract (Difco) 3 g, Bacto-peptone (Difco) 5 g, malt extract (Oxoid) 3 g, glucose 10 g and saccharose 340 g. After autoclaving 2 ml of sterile 2.5M $MgCl_2$ solution and 25 ml of 20% glycine are added.

SGYEME As YEME, but the amount of saccharose was 110 g per litre. To prepare protoplasts the amount of 20% glycine varies from 12 ml to 50 ml per litre depending on the strain used.

YM-agar Bacto Yeast malt extract agar, ISP-medium 2, Difco; 38 g/litre.

ISP4 Bacto ISP-medium 4, Difco; 37 g/litre.

R2YE Hopwood *et al.*, (1985 p. 236)

LB Sambrook *et al.*, (1989, 3:A.1)

E1 Per litre: Glucose 20 g, starch 20 g, Farmamedia 5 g, yeast extract 2.5 g, $K_2HPO_4 \cdot 3H_2O$ 1.3 g, $MgSO_4 \cdot 7H_2O$ 1 g, NaCl 3 g, $CaCO_3$ 3 g. Tap water is added to 1 litre and pH is adjusted to 7.4.

TE Tris-HCl-buffer, pH 8: 10 mM, EDTA, pH 8: 1 mM

20*SSC Per litre: NaCl 175.3 g, Na-citrate 88.2 g. pH is adjusted to 7 with NaOH.

DENHARDT SOLUTION (Sambrook *et al.*, 1989, 3:B.1)
A 50* basic solution is prepared, which contains Ficoll 5 g, polyvinyl pyrrolidone 5 g, BSA (bovine serum albumin) 5 g. Distilled water is added to 500 ml and sterilized by filtrating.

Example 1. Cloning and characterization of the genes included in the anthracycline biosynthesis of *Streptomyces nogalater*

1.1 Preparing of gene bank and cloning of anthracycline genes from *S. nogalater*.

Isolation of the total DNA from *Streptomyces nogalater*

S. nogalater (ATCC 27451) mycelia were cultivated for about 3 days in 50 ml of TSB medium, wherein 0.5% glycine had been added at 28 °C vigorously shaking. The mycelia were pelleted and the supernatant was discarded. The pellet was suspended into 10 ml of lysis buffer (15 % saccharose, 25 mM Tris, pH 8.0, 25 mM EDTA and 5 mg/ml of lysozyme) and incubated for 15 min at 37 °C. 1 mg of proteinase K and

1 ml of 10% SDS were added while stirring. The mixture was incubated at once for 15 min at 70 °C. The lysed pellet was subsequently cooled in ice, 1 ml of 3 M Na-acetate (pH 6.0) was added and kept for a few minutes on ice bath. 5 ml of phenol balanced with 0.1 M Tris was added and stirred by turning the tube around. The phases were sentrifuged apart and the water phase was further extracted with 5 ml of chloroform. DNA was subsequently precipitated by adding 10 ml of isopropanol. DNA was spinned cautiously around a Pasteur pipette being closed by flaming, washed by dipping into 70% ethanol and DNA was loosened onto the wall of the tube. DNA was dissolved in 5 ml of TE-buffer and treated with RNase (25 µl of 10 mg/ml DNase free RNase) for about 30 min at 37 °C. The phenol and chloroform extractions were repeated. DNA was subsequently reprecipitated with isopropanol and washed as above. Finally DNA was dissolved in 1 ml of TE-buffer and it was used for subsequent steps.

Southern hybridization

The *actI* probe was the 0.8 kb *BglII*-fragment obtained from the plasmid pIJ2345 and the *acm* probe the 3 kb *Bam*HI-fragment obtained from the plasmid pACM5 (Niemi *et al.*, 1994). The plasmids were isolated at mini-scale (Magic Minipreps reagent series of Promega) and the probe fragments were isolated by preparative agarose gel electrophoresis after digesting them first with *BglII* and with *Bam*HI, respectively. The probes were then labeled with 50 µCi of [α^{32}-P]CTP by nick-translation (Nick translation labeling reagent series of Boehringer Mannheim).

The total DNA preparations isolated as described above were digested with *Eco*RI enzyme and fractionated with agarose gel electrophoresis. The fractionated DNA was transferred from the gel to Hybond N membrane (Amersham) using the Vacugene apparatus (LKB 2016, Pharmacia LKB Biotechnology) according to the instructions of use. DNA was fastened into the membrane by incubating for 3 min in UV light.

The membranes were hybridized in 10 ml of hybridization solution (1% SDS, 1M NaCl, 5* Denhardt's solution, 100 µg/ml denatured carrier DNA (DNA from calf thymus, Boehringer Mannheim) at 65 °C in a hybridization oven (HB-1D Hybridiser, Techne) for about 6 h, whereafter at least 100 ng of labeled probe-DNA was added

into the hybridization tube and the inkubation was continued for further about 12 h. After this the membranes were washed at 65 °C for 2*30 min in a wash solution (2*SSC, 1% SDS or 0.2*SSC, 0.1% SDS). Autoradiography was effected by superimposing the membrane coated with a plastic film and the autoradiography film. Exposure lasted about 1 to 3 days.

Preparing of the gene bank from *S. nogalater* DNA

40 µg of DNA was incubated in the digestion buffer (10*A, Boehringer Mannheim) in the presence of 2.4 units of *Sau*3A (Boehringer Mannheim) for 5 min at 37 °C and the reaction was stopped by adding phenol. After phenol treatment DNA was purified with ethanol precipitation. DNA-fragments so obtained were run at preparative agarose gel electrophoresis (0.3 % LGT, low gelling temperature). DNA, which was 20 kb or bigger, was taken from the gel by cutting and purified by phenolization from the agarose. A commercial phage vector, λ EMBL 4, *Bam*HI fragments (Amersham International plc, Amersham UK) were treated with alkaline phosphatase (CIAP, calf intestinal alkaline phosphatase, Promega) according to the instructions of the manufacturer. The insert DNA (*Sau*3A fraction) and vector so obtained were ligated by incubating for 2 h at room temperature and for 2 h at 14 °C in the presence of T4-DNA ligase (Promega) according to the recommendation of the manufacturer. The ligation mixture was packed to λ-particles using the Packagene reagent series (Promega Biotech) according to the manufacturer's instructions. *Escherichia coli* strain GM2163 was used as the host. The cells were prepared for infection according to the packing instructions and cells infected with the packing mixture were spread onto plates according to Promega's instructions.

Isolation and mapping of hybridizing clones

Phage DNA from plates with about 4000 plaques/plate was transferred to a membrane (Colony/Plaque Screen, New England Nuclear) according to the manufacturer's instructions. The membranes were hybridized as described above. Plaques which gave a signal in autoradiography, were picked up and the phages were eluted from them by incubating a plaque in 0.5 ml of SM-buffer for 2 hours. Because the plaque plates

were dense, the plaques were purified by infecting them into the host strain LE392 (Promega) and hybridizing as above.

From the purified clones phage DNA was prepared in 20 ml scale by infecting the LE392 cells according to Promega's packing instructions. The DNA so obtained was digested with various restriction endonucleases to map the clones (Sambrook *et al.*, 1989) and by hybridizing with different probes. The restriction map so obtained is given in Fig. 2.

Transfer of the DNA fragments to *S. lividans* and detection of new compounds

The fragment shown in the restriction map (Fig. 2) was transferred into *S. lividans* as *Eco*RI-fragments (pSY1 and pSY6) or as a *Bgl*II-fragment (pSY15). λ-clones were digested with *Eco*RI or *Bgl*II-restriction enzyme and ligated to a plasmid made linear with the same enzyme and was transformed by electroporation into *E. coli* or by protoplast transformation into *S. lividans*. Most of the inserts were first cloned into the plasmid pUC19 amplifying in *E. coli*, whereby as a host *E. coli* strain XL1-Blue was used. pSY15 was cloned directly into the *S. lividans* strain TK24. *E. coli* was used because by that way smaller amounts of phage-DNA could be used. The transformation efficacy of *E. coli* was $2*10^8$ transformants/μg DNA, when *E. coli* Pulser Apparatus-electroporation device (Bio-Rad) was used with the following settings (200 Ohm, 25 μF, 1.4 kV). For electroporation the cells were treated as described in Dower, W.J. *et al.* (1988), and 0.1 cm cuvettes of Bio-Rad were used in transformation, the cell volume was 20 μl.

S. lividans strain TK24 was used as an intermediate host as the expression was believed to be successful only in *S. galilaeus* strains. *S. galilaeus* is not at all transformable with DNA propagated in *E. coli*. Only the plasmid pSY15 caused modification in TK24 strain, which was noticed as brown colour on the ISP4 plate, when TK24 is normally rather colourless or blue. Only the TK24 strain carrying the plasmid pSY15 caused formation of coloured products in the E1-medium well suited for the production of anthracyclines. On the basis of thin layer chromatography the products of the recombinant strain TK24/pSY15 seemed to be alike to but not identical

with those produced by the mutant H036 (Ylihonko *et al.*, 1994) producing the methyl ester of aklanone acid. With the eluent toluene:ethyl acetate:methanol:formic acid (50:50:15:3) the following R_f-values were obtained for these products:

TK24/pSY15: 0.66; 0.60; 0.50
H036: 0.67; 0.62; 0.51.

These characteristics were confirmed to come from the pSY15 plasmid by retransforming the plasmid to *S. lividans* TK24 strain. The transformants so obtained were also able to produce anthracycline precursors. When the recombinant strain was cultivated in E1 medium without selection pressure of the plasmid strain caused by thiostrepton, the production of new compounds decreased.

1.2 Localizing the PKS-genes

Sequencing of the hybridizing fragment

From the *Eco*RI-digest a 2 kb *actI* hybridizing fragment was obtained and it was sequenced. About 2 kb of DNA to the right according to the map (Fig. 2) was additionally sequenced. For sequencing 31 clones were prepared from restriction enzyme digestion sites to the vectors pUC18 and pUC19, being linearized with corresponding enzymes.

To isolate plasmids for the sequencing reactions Magic/Wizard™ Minipreps DNA Purification System kit of Promega was used. *E. coli* XL1-Blue cells were cultivated overnight in 3 ml of LB-medium which contained 50 µg/ml of ampicillin, and the plasmids were isolated according to the manufacturer's instructions.

DNA-sequencing was performed by using dideoxy chain termination method. For the sequencing reactions Deaza G/A ^{T7}Sequencing™ Mixes (Pharmacia) and TaqTrack® Sequencing Systems, Deaza (Promega) sequencing reagent series were used. Denaturation was always performed according to the instructions in the Pharmacia kit. (Method C). When using the Pharmacia kit the primers were ligated according to the

Method C given in the instructions (Standard Annealing of Primer to Double-Stranded Template). When using the Promega kit the item "Sequencing Protocol Using Direct Incorporation" of the manufacturer's instructions was followed. Deviating from the primer ligation temperature (37 °C) recommended by the manufacturer the temperature of 45 °C was used to avoid the secondary structures caused by the high GC-content. The temperature was kept thereafter at 45 °C until the end of the reaction. As a radioactive label [α^{35}S]dATP (NEN Products Boston, MA) was used. Most of the PKS-domain was sequenced with a universal primer (5'-d(GTTTTCCCAGTCAC-GAC)-3') and with a reverse primer (5'-d(CAGGAAACAGCTATGAC)-3' (pUC/M13 17-mer Primers, Promega). When sequencing the longest fragments (500-600 bp) of the domain, and in order to define the sequences of such restriction sites which could not be "passed", six specific primers were used. The primers were prepared at the Department of Bioorganic Chemistry in the University of Turku.

The sequencing gels were run by the Macrophor-system of Pharmacia, using a 4% thickness gradient gel. Running conditions: current 20 mA, voltage 2500 V.

Sequence analysis

From the PKS domain the DNA fragment with about 4134 bases (as given in the sequence listing) was sequenced, the analysis of which was performed by GCG-software (Genetics Computer Group, GCG Package, Wisconsin USA). With the subprogram CODONPREFERENCE the open reading frames were sought from the sequence. The reading frames obtained were translated to the amino acid sequence and with the TFASTA-subprogram homologies to known sequences were sought.

According to the CODONPREFERENCE program the 4134 base DNA fragment as sequenced had altogether three open reading frames (ORF1, ORF2, ORF3) (ORF 1 is the fragment 359-1651 in SEQ ID NO:1 of the sequence listing, ORF2 is the fragment 1648-2877 in the SEQ ID NO:4, and ORF3 is the fragment 2937-3197 in the SEQ ID NO:1). In the beginning of each open reading frame a possible ribosome binding site was found (RBS). The functions of the genes were concluded by comparing the amino acid sequences translated from their base sequences to known sequences. So the

following similarities with the open reading frames of actinorhodin and tetracenomycin PKS domains were obtained: ORF1 (80%, 81%), ORF2 (74%, 77%), ORF3 (62%, 62%), and on the basis of this we present the following functions to said genes: ORF1 is ketoacylsynthase; ORF2 is the factor which effects on the chain length; ORF3 is an acyl carrier protein. These three open reading frames are needed for a functional polyketide synthase.

Upstream of the PKS domain about 6 kb DNA fragment was sequenced (kb = 1000 bases). In this domain the following gene activities have been recognized on the basis of the sequence: (Fig. 2): regulatory genes, mono-oxygenase, methyl transferase and ketoreductase.

Example 2. Transfer of the genes into the strain *S. galilaeus* ATCC 31615 and mutants thereof

Plasmid pSY15 was isolated from *S. lividans* strain TK24 and transformed into *S. galilaeus* mutant H039 and the DNA isolated therefrom further into other *S. galilaeus* mutants. The method used in the transformation of the *S. galilaeus* strain being modified from the transformation method used in the transformation of *S. lividans* has been described earlier (Ylihonko, K., Pro gradu-thesis, University of Turku, 1986). For preparing protoplasts the cells were grown in SGYEME, to which 0.8% saccharose had been added. The plasmids were transformed successfully first to the mutant H039, whereby with 2 µg of plasmid-DNA about 10 transformants were obtained. Because of a strong restriction barrier *S. galilaeus* is weakly transformable with foreign DNA but the transformation efficacy increases manyfold if the plasmid has been isolated from a *S. galilaeus* strain.

H039-transformants were first cultivated for about 5 days on an ISP4 plate, whereto thiostreptone had been added. The mycelium was inoculated in 50 ml of TSB nutrient broth (5 µg/ml of thiostreptone added) and grown in a shaker for 5 days. The plasmid was isolated as described above and transformed into other mutants. Usually 200 to

500 ng of plasmid was used per one transformation, whereby 10 to 100 transformants were obtained.

After regeneration the transformed mutant strains were spread onto ISP4 plates, wherefrom the mycelium was further transferred to E1 nutrient medium. To retain the plasmid thiostrepton was added to all nutrient media. E1 mycelium was incubated in a shaker (330 rpm, 30 °C) and production was followed by taking after 3 days a 0.5 ml sample of the mycelium daily for 3 to 5 days. The sample was buffered to pH 7 with phosphate buffer and extracted with methanol–toluene mixture (1:1). In addition, part of the samples were acidified with 1M HCl solution and extracted into toluene–methanol. In E1–cultivations both mutants and the *S. galilaeus* wild strain were used as controls. By comparing the products on TLC the effects of the plasmid on the production were seen.

The *S. galilaeus* mutants used in transformations are listed above. Plasmid pSY15 complemented, i.e. restored the producing ability of anthracyclines or precursors thereof in the following mutants: H028, H061 and JH003. It did not affect the production profile of the mutants H036 and H039 to any appreciable extent. JH003, which does not produce coloured compounds in the conditions used, has been mutated from the strain H054 and the transformant JH003/pSY15 was compared to the strain H054. H028 is also a non–producing mutant, which was obtained by mutating the wild strain *S. galilaeus* ATCC 31615. So the wild strain was used as the control of the transformant H028/pSY15. Using the eluent toluene:ethyl acetate:methanol:formic acid (50:50:15:3) the following R_f–values were obtained for the transformants and the host strains used as controls.

H028/pSY15: (0.69); 0.61; 0.58; 0.01
JH003/pSY15: 0.59; 0.50; 0.46; 0.35
H061/pSY15: (0.69); 0.61; 0.58; 0.06; 0.01
S. galilaeus ATCC 31615: 0.23; 0.14; 0.11
H054: 0.65; 0.60; 0.53; 0.48.
H061: 0.50 (acid).

The product isolated in small scale was hydrolyzed by heating in 1M hydrochloric acid at 80 °C for 0.5 h. After hydrolysis the following R_f-values were obtained for the aglycons or precursors thereof:

H028/pSY15: 0.61

JH003/pSY15: 0.61

H061/pSY15: 0.61.

Because all these mutants used have originally been produced from a *S. galilaeus* wild strain, aklavinone was used as comparison, being the aglycone of aclacinomycins produced by *S. galilaeus*. In the eluent used the R_f-value 0.69 was obtained for aklavinone. In the products of transformants small amounts of aklavinone were also detected.

Example 3. Production of anthracycline precursors

3.1 Production of TK24/pSY15 products

Ten 250 ml erlenmeyer-flasks each containing 60 ml of E1-medium were inoculated with 1 ml aliquots of the strain TK24/pSY15. The flasks were incubated in a shaker at 330 rpm at the temperature of 30 °C for about 3 days. From the finished mycelia production was confirmed by extracting 0.5 ml samples with a mixture of methanol and toluene (1:1). The products were compared to the standard by thin layer chromatography.

The flasks were emptied into two 400 ml centrifuge tubes and centrifuged for 10 min at 3000 rpm. The supernatant was recovered. The precipitate was suspended by adding to each tube 50 ml of methanol. The tubes were recentrifuged for 10 min at 3000 rpm. The methanol solution was added into the supernatant. The precipitate was discarded. The solution was extracted with 2 * 100 ml of chloroform, whereby a strongly orange-yellow chloroform solution was obtained. The water phase was discarded.

Chloroform was evaporated on a water bath in a rotary evaporator. The orange-yellow, dry product was dissolved in 2 ml of chloroform.

The chloroform solutions were pipetted into a chromatography column of glass, equipped with a glass sinter, having a diameter of 2 cm and containing about 5 cm of silica suspended in chloroform (Kieselgel 60, Merck). The column was eluted with 2.5 ml aliquots of chloroform. Each fraction was collected into a separate test tube. Samples of each fraction were dropped on a thin layer and compared to the standards. Fractions containing individual compounds were pooled and evaporated into dryness.

NMR-spectra of pure compounds were determined and the compounds were identified by comparing the spectra with analogical compounds. In Fig. 4 the H-NMR-spectrum of Compound I is given.

3.2 Production of an aglycone in the strain H028/pSY15

Ten 250 ml erlenmeyer-flasks each containing 60 ml of E1-medium were inoculated with 1 ml aliquots of the strain H028/pSY15. The flasks were incubated in a shaker at 330 rpm at the temperature of 30 °C for about 4 days. From the finished mycelia production was confirmed by extracting 0.5 ml samples with a mixture of methanol and toluene (1:1). The products were compared to the standards by thin layer chromatography.

The flasks were emptied into two 400 ml centrifuge tubes and centrifuged for 10 min at 3000 rpm. The supernatant was recovered. The precipitate was suspended by adding to each tube 50 ml of methanol. The tubes were recentrifuged for 10 min at 3000 rpm. The methanol solution was added into the supernatant. The precipitate was discarded. The solution was extracted with 2 * 100 ml of chloroform, whereby a strongly yellow chloroform solution was obtained. The water phase was discarded.

Chloroform was evaporated on a water bath in a rotary evaporator. The yellow, dry product was dissolved in 2 ml of chloroform.

The chloroform solutions were pipetted into a chromatography column of glass, equipped with a glass sinter, having a diameter of 2 cm and containing about 5 cm of silica suspended in chloroform (Kieselgel 60, Merck). The column was eluted with 2.5 ml aliquots of chloroform. Each fraction was collected into a separate test tube. Samples of each fraction were dropped on a thin layer and compared to the standards. Fractions containing individual compounds were pooled and evaporated into dryness.

NMR-spectra of pure compounds were determined and the compounds were identified by comparing the spectra with analogical compounds. In Fig. 5 the H-NMR-spectrum of auramycinone (Compound II) is given.

Example 4. Biotransformation of hybrid products

4.1 Biotransformation of auramycinone in strain JH003

A 250 ml erlenmeyer-flask containing 60 ml of E1-medium was inoculated with 1 ml of strain JH003. The flask was incubated in a shaker at 330 rpm at the temperature of 30 °C for about 3 days. After two day's cultivation about 2 mg of auramycinone was added into the flask. At 24 hours from this the production was confirmed by extracting a 0.5 ml sample with the mixture of methanol and toluene (1:1). The products were compared to the standard by thin layer chromatography.

The flask was emptied into two 60 ml centrifuge tube and centrifuged for 10 min at 3000 rpm. The supernatant was recovered. The precipitate was suspended by adding to each tube 10 ml of methanol. The tubes were recentrifuged for 10 min at 3000 rpm. The methanol solution was added to the supernatant. The precipitate was discarded. The pooled solution was extracted with 2 * 20 ml of chloroform, whereby a strongly yellow chloroform solution was obtained. The water phase was discarded.

Chloroform was evaporated on a water bath in a rotary evaporator. The yellow, dry product was dissolved in chloroform. On the basis of TLC the product was found to correspond to the products of the strain JH003/pSY15 (cf. Example 5.2).

Example 5. Production of hybrid anthracyclines

5.1 Production of auramycinone-rhodosamine-deoxyfucose in strain H028/pSY15

Ten 250 ml erlenmeyer-flasks each containing 60 ml of E1-medium were inoculated with 1 ml aliquots of the strain H028/pSY15. The flasks were incubated in a shaker at 330 rpm at the temperature of 30 °C for about 4 days. From the finished mycelia production was confirmed by extracting 0.5 ml samples with a mixture of methanol and toluene (1:1). The products were compared to the standard by thin layer chromatography.

The flasks were emptied into two 400 ml centrifuge tubes and centrifuged for 10 min at 3000 rpm. The supernatant was recovered. The precipitate was suspended by adding to each tube 50 ml of methanol. The tubes were recentrifuged for 10 min at 3000 rpm. The methanol solution was added into the supernatant. The precipitate was discarded. The pooled solution was extracted with 2 * 100 ml of chloroform, whereby a strongly yellow chloroform solution was obtained. The water phase was discarded.

Chloroform was evaporated on a water bath in a rotary evaporator. The yellow, dry product was dissolved in 2 ml of chloroform.

The chloroform solutions were pipetted into a chromatography column of glass, equipped with a glass sinter, having a diameter of 2 cm and containing about 5 cm of silica suspended in chloroform (Kieselgel 60, Merck). The column was eluted with 2.5 ml aliquots of chloroform. Each fraction was collected into a separate test tube. Samples of each fraction were dropped on a thin layer and compared to the standards. Fractions containing individual compounds were pooled.

NMR-spectra of pure compounds were determined and the compounds were identified by comparing the spectra with analogical compounds. In Fig. 6 the H-NMR-spectrum of auramycinone-rhodosamine-deoxyfucose (Compound III) is given.

5.2 Production of auramycinone-rhodinose-deoxyfucose in strain JH003/pSY15

Ten 250 ml erlenmeyer-flasks each containing 60 ml of E1-medium were inoculated with 1 ml aliquots of the strain JH003/pSY15. The flasks were incubated in a shaker at 330 rpm at the temperature of 30 °C for about 4 days. From the finished mycelia production was confirmed by extracting 0.5 ml samples with a mixture of methanol and toluene (1:1). The products were compared to the standard by thin layer chromatography.

The flasks were emptied into two 400 ml centrifuge tubes and centrifuged for 10 min at 3000 rpm. The supernatant was recovered. The precipitate was suspended by adding to each tube 50 ml of methanol. The tubes were recentrifuged for 10 min at 3000 rpm. The methanol solution was added into the supernatant. The precipitate was discarded. The pooled solution was extracted with 2 * 100 ml of chloroform, whereby a strongly yellow chloroform solution was obtained. The water phase was discarded.

Chloroform was evaporated on a water bath in a rotary evaporator. The yellow, dry product was dissolved in 2 ml of chloroform.

The chloroform solutions were pipetted into a chromatography column of glass, equipped with a glass sinter, having a diameter of 2 cm and containing about 5 cm of silica suspended in chloroform (Kieselgel 60, Merck). The column was eluted with 2.5 ml aliquots of chloroform:methanol 100:10. Each fraction was collected into a separate test tube. Samples of each fraction were dropped on a thin layer and compared to a standard. Fractions containing individual compounds were pooled and evaporated into dryness.

NMR-spectra of pure compounds were determined and the compounds were identified by comparing the spectra with analogical compounds. In Fig. 7 the H-NMR-spectrum of auramycinone-rhodinose-deoxyfucose is given.

Example 6. Characterization of the products

6.1 HPLC-runs

The retention times of the compounds were determined at RP-18-column, with an eluent acetonitrile:methanol:potassium dihydrogen phosphate buffer (8.00 g/l, pH 3.0) 5:2:3. The retention times of the compounds are: I: 4.63, II: 3.52, III: 4.09 and IV: 7.26. The structures of the compounds I - IV are given in the Scheme I.

6.2 NMR-spectra of the compounds

H-NMR-spectra of some of the TK24/pSY15, H028/pSY15 and JH003/pSY15 products were determined by Brüker 400 MHz NMR spectrometer in deuterium-chloroform. The spectra given by the compounds were compared to the spectra of known compounds, e.g. aclarubicin. The spectra obtained are given in Figs. 4 to 7.

In all of the compounds the hydrogens in 1, 2 and 3-positions bound to each other and with same transitions are found. The singlet corresponding to the hydrogen in 11-position was found in all compounds with the same transition. Additionally, the peaks given by the two aromatic hydroxyls can be seen. On the basis of the peaks of these six hydrogens the aromatic chromophore moieties are similar, and correspond e.g. the chromophore of aklavinone.

In all of the compounds a singlet of the size of three hydrogens is found at about 3.7 ppm corresponding to the methyl of methyl ester. Another singlet is found in all compounds at about 3.8 ppm, which corresponds to the 10-position hydrogen. The integral of this is of the size of one hydrogen in auramycinone and its glycosides and of the size of two hydrogens in Compound I. According to this Compound I suits to be a compound in which the fourth ring has not been closed.

The region 4.7 to 6 ppm has in anthracyclines and in compounds related thereto hydrogens at 7-position and 1-position of the sugars. Auramycinone has in this region one peak, Compounds III and IV have three peaks, but in Compound I there are no peaks in this region. According to this auramycinone has no sugars and Compounds

III and IV have two sugars, whereas Compound I has no hydrogens in this region which suits with the keto–form at position 7.

Auramycinone and its glycosides have a three hydrogen singlet between 1.39 and 1.47 ppm. This suits to be the methyl group of position 13, which is not bound to other hydrogens. This item distinguishes these compounds from aklavinone and its glycosides, wherein the side chain is ethyl.

The 8–position CH_2–hydrogens of auramycinone and its glycosides give one doublet at 2.2 ppm and a double doublet at 2.6 ppm. In addition, in the spectra of Compounds III and IV peaks corresponding to their sugars are found.

The H–NMR results match well with the structures given in the Figures.

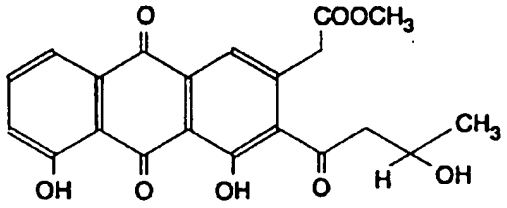
Deposited microorganisms

The following microorganism was deposited in Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Mascheroder Weg 1 b, D–38124 Braunschweig, Germany

Microorganism	**Deposition number**	**Deposition date**
Streptomyces lividans TK24/pSY15	DSM 9436	15 September 1994

Scheme I Structural formulas of the compounds obtained

I



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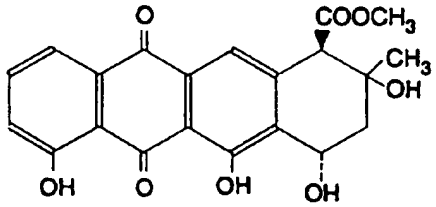

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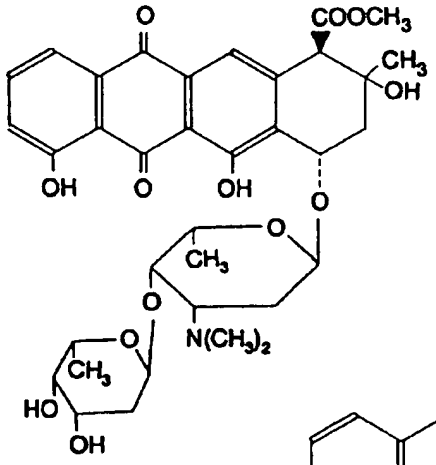
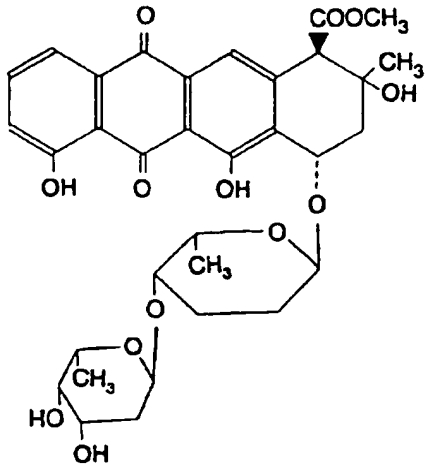


IV



References

Arrowsmith, T.J., Malpartida, F., Sherman, D.H., Birch, A., Hopwood, D.A. and Robinson, J.A. 1992. Characterization of *actI*-homologous DNA encoding polyketide synthase genes from monensin producer *Streptomyces cinnamonensis*. Mol. Gen. Genet. **234**: 254–264

Bhuyan, B.K. and Dietz, A. 1965. Fermentation, taxonomic, and biological studies of nogalamycin. Antimicrob. Agents Chemother. **1965**:836–844.

Bibb, M.J., Sherman, D.H., Omura, S. and Hopwood, D.A. 1994 Cloning, sequencing and deduced functions of a cluster of Streptomyces genes probably encoding biosynthesis of the polyketide antibiotic frenolicin. Gene

Bibb, M.J. Biro, S., Motamedi, H., Collins, J.F. and Hutchinson, C.R. 1989. Analysis of the nucleotide sequence of the *Streptomyces glaucescens tcmI genes* provides key information about the enzymology of polyketide antibiotic biosynthesis. EMBO J. 8: 2727–2736.

Dower W.J., Miller, J.F. and Ragsdale, C.W. 1988. High efficiency transformation of *E.coli* by high voltage electroporation. Nucleic Acids Research **16**:6127–6145.

Fernandez–Moreno, M.A., Martinez, E., Boto, L., Hopwood, D.A. and Malpartida, F. 1992. Nucleotide sequence and deduced functions of a set of cotranscribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin. J.Biol.Chem. **267**: 19278–19290

Floss, H.G. (1987) Hybrid antibiotics – the contribution of the new gene combinations. Trends in Biotech. **5**:111–115.

Fujivara, A. and Hoshino, T. 1986. Anthracycline antibiotics. CRC critical reviews in biotechnology. **3**:2:133–157.

Gräfe, U., Dornberger, K., Wagner, C and Eckardt, K. 1989. Advances in bioconversion of anthracycline antibiotics. Biotech. Adv. **7**:215–239.

Hallam, S.E., Malpartida, F. and Hopwood, D.A. 1988. Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. Gene **74**:305–320.

Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, H.M. Kieser, D.J. Lydiate, C.P. Smith, J.M. Ward, and H. Schrempf. 1985. Genetic manipulations of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.

Katz, L., and Donadio, S. 1993. Polyketide synthesis: prospects for hybrid antibiotics. Annu. Rev. Microbiol. **47**:875–912.

Kim, E-S., Bibb, M.J., Butler, M.J., Hopwood, D.A. and Sherman, D.H. 1994. Nucleotide sequence of the oxytetracycline (otc) polyketide synthase genes from *Streptomyces rimosus*. Gene **141**:141-142.

Malpartida, F. and Hopwood, D.A. 1984. Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature **309**:462-464.

Malpartida, F., S.E. Hallam, H.M. Kieser, H. Motamedi, C.R. Hutchinson, M.J. Butler, D.A. Sugden, M. Warren, C.McKillop, C.R. Bailey, G.O. Humphreys, and D.A. Hopwood. 1987. Homology between *Streptomyces* genes coding for synthesis of different polyketides used to clone antibiotic synthesis genes. Nature (London) 325:818-821.

McDaniel, R. , S. Ebert-Khosla, D.A. Hopwood, and C. Khosla. 1993. Engineered biosynthesis of novel polyketides. Science **262**:1546-1550.

Niemi, J., K. Ylihonko, J. Hakala, R. Pärssinen, A. Kopio, and P. Mäntsälä. 1994. Hybrid anthracycline antibiotics: production of new anthracyclines by cloned genes from *Streptomyces purpurascens* in *Streptomyces galilaeus*. Microbiol. **140**:1351-1358.

Sambrook, J., E.F. Fritsch, and T.Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Shen, B. and Hutchinson, R. 1993. Enzymatic synthesis of a bacterial polyketide from acetyl and malonyl Coenzyme A. Science **262**:1535-1540.

Sherman, D.H., Malpartida, F., Bibb, M.J., Kieser, H.M. and Hopwood, D.A. 1989. Structure and deduced function of the granaticin-producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tü22. EMBO J. **8**: 2717-2725.

Ward, J.M, G.R. Janssen, T. Kieser, M.J. Bibb, M.J. Buttner and M.J. Bibb. 1986. Construction and charaterization of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase from Tn5 as indicator. Mol. Gen. Genet. **203**:468-478.

Ylihonko, K., J. Hakala, J. Niemi, J. Lundell and P. Mäntsälä. 1994. Isolation and characterization of aclacinomycin A-nonproducing *Streptomyces galilaeus* (ATCC 31615) mutants. Microbiol. **140**:1359-1365.

Yu, T.-W., Bibb, M.J., Revill, W.P. and Hopwood, D.A. 1994. Cloning, sequencing and analysis of the griseusin polyketide synthase gene cluster from *Streptomyces griseus*. J. Bacteriol. **176**:2627-2634.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Galilaeus Oy
(B) STREET: Elinantie 2 A 9
(C) CITY: Turku
(E) COUNTRY: Suomi
(F) POSTAL CODE (ZIP): FIN-20510

(ii) TITLE OF INVENTION: Process for producing anthracyclines and intermediates thereof

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3252 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: *Streptomyces nogalater* ATCC 27451

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 359..1651
(D) OTHER INFORMATION: /note= "ORF1"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2937..3197
(D) OTHER INFORMATION: /note= "ORF3"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1648..1651
(D) OTHER INFORMATION: /note= "overlapping sequence in ORF1 and ORF2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GTCCGCGCAC AGGAACTTCA AGACGATCAA AGCCCCTAGT GAAGGGCATC TTCGACGA        358

ATG AAG GAA TCC ATC AAC CGT CGC GTG GTC ATC ACC GGA ATA GGG ATC        406
Met Lys Glu Ser Ile Asn Arg Arg Val Val Ile Thr Gly Ile Gly Ile
  1               5                  10                  15

GTC GCG CCC GAT GCC ACC GGG GTG AAA CCG TTC TGG GAT CTG CTG ACG        454
Val Ala Pro Asp Ala Thr Gly Val Lys Pro Phe Trp Asp Leu Leu Thr
            20                  25                  30

GCC GGT CGC ACC GCG ACC CGG ACC ATC ACC GCC TTC GAT CCC TCT CCG        502
Ala Gly Arg Thr Ala Thr Arg Thr Ile Thr Ala Phe Asp Pro Ser Pro
        35                  40                  45

TTC CGT TCC CGC ATC GCC GCG GAA TGC GAT TTC GAC CCG CTT GCC GAA        550
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    50                  55                  60

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65                  70                  75                  80

GCG GTC GTC AGC GCC CGG GAA AGC CTG GAG GAC AGC GGA CTC GAC CTC        646
Ala Val Val Ser Ala Arg Glu Ser Leu Glu Asp Ser Gly Leu Asp Leu
                85                  90                  95

GGC GCC CTG GAC GCC TCC CGC ACC GGC GTG GTC GTC GGC AGC GCG GTC        694
Gly Ala Leu Asp Ala Ser Arg Thr Gly Val Val Val Gly Ser Ala Val
            100                 105                 110

GGC TGC ACC ACG AGC CTG GAA GAG GAG TAC GCG GTC GTC AGC GAC AGC        742
Gly Cys Thr Thr Ser Leu Glu Glu Glu Tyr Ala Val Val Ser Asp Ser
        115                 120                 125

GGC CGG AAC TGG CTG GTC GAC GAC GGC TAC GCC GTA CCG CAC CTA TTC        790
Gly Arg Asn Trp Leu Val Asp Asp Gly Tyr Ala Val Pro His Leu Phe
    130                 135                 140

GAC TAC TTC GTG CCC AGC TCC ATC GCC GCC GAG GTG GCA CAC GAC CGC        838
Asp Tyr Phe Val Pro Ser Ser Ile Ala Ala Glu Val Ala His Asp Arg
145                 150                 155                 160

ATC GGC GCG GAG GGC CCC GTC AGC CTC GTG TCG ACC GGG TGC ACC TCG        886
Ile Gly Ala Glu Gly Pro Val Ser Leu Val Ser Thr Gly Cys Thr Ser
                165                 170                 175

GGC CTG GAC GCC GTG GGC CGC GCG GCC GAC CTG ATC GCC GAG GGA GCG        934
Gly Leu Asp Ala Val Gly Arg Ala Ala Asp Leu Ile Ala Glu Gly Ala
            180                 185                 190

GCG GAT GTG ATG CTG GCC GGT GCG ACC GAG GCG CCC ATC TCC CCC ATC        982
Ala Asp Val Met Leu Ala Gly Ala Thr Glu Ala Pro Ile Ser Pro Ile
        195                 200                 205

ACC GTG GCG TGC TTC GAT GCC ATC AAG GCG ACC ACC CCC CGC AAC GAC       1030
Thr Val Ala Cys Phe Asp Ala Ile Lys Ala Thr Thr Pro Arg Asn Asp
    210                 215                 220

ACG CCC GCC GAG GCG TCC CGT CCG TTC GAC CGC ACC AGG AAC GGG TTC       1078
Thr Pro Ala Glu Ala Ser Arg Pro Phe Asp Arg Thr Arg Asn Gly Phe
225                 230                 235                 240
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GTA CTC GGC GAG GGC GCT GCC GTG TTC GTC CTG GAG GAG TTC GAA CAC      1126
Val Leu Gly Glu Gly Ala Ala Val Phe Val Leu Glu Glu Phe Glu His
                245                 250                 255

GCG CGC CGC CGG GGC GCG CTC GTG TAC GCG GAG ATC GCC GGG TTC GCC      1174
Ala Arg Arg Arg Gly Ala Leu Val Tyr Ala Glu Ile Ala Gly Phe Ala
            260                 265                 270

ACT CGC TGC AAC GCC TTC CAC ATG ACC GGT CTG CGC CCG GAC GGG CGG      1222
Thr Arg Cys Asn Ala Phe His Met Thr Gly Leu Arg Pro Asp Gly Arg
        275                 280                 285

GAG ATG GCG GAG GCC ATC GGG GTG GCG CTC GCC CAG GCG GGC AAG GCG      1270
Glu Met Ala Glu Ala Ile Gly Val Ala Leu Ala Gln Ala Gly Lys Ala
    290                 295                 300

CCG GCT GAC GTC GAC TAC GTC AAC GCC CAC GGT TCC GGC ACC CGG CAG      1318
Pro Ala Asp Val Asp Tyr Val Asn Ala His Gly Ser Gly Thr Arg Gln
305                 310                 315                 320

AAT GAC CGT CAC GAG ACG GCG GCC TTC AAG CGC AGT CTC GGC GAC CAC      1366
Asn Asp Arg His Glu Thr Ala Ala Phe Lys Arg Ser Leu Gly Asp His
                325                 330                 335

GCC TAC CGG GTC CCG GTC AGC AGC ATC AAA TCC ATG ATC GGG CAC TCG      1414
Ala Tyr Arg Val Pro Val Ser Ser Ile Lys Ser Met Ile Gly His Ser
            340                 345                 350

CTG GGC GCG ATC GGC TCC CTG GAG ATC GCC GCC TCC GTG CTG GCC ATC      1462
Leu Gly Ala Ile Gly Ser Leu Glu Ile Ala Ala Ser Val Leu Ala Ile
        355                 360                 365

ACA CAC GAC GTG GTG CCG CCC ACC GCC AAT CTG CAC GAG CCG GAT CCC      1510
Thr His Asp Val Val Pro Pro Thr Ala Asn Leu His Glu Pro Asp Pro
    370                 375                 380

GAG TGC GAT CTG GAC TAC GTG CCG CTG CGG GCG CGT GCG TGC CCG GTG      1558
Glu Cys Asp Leu Asp Tyr Val Pro Leu Arg Ala Arg Ala Cys Pro Val
385                 390                 395                 400

GAC ACG GTG CTC ACG GTG GGC AGC GGG TTC GGC GGT TTC CAG AGC GCC      1606
Asp Thr Val Leu Thr Val Gly Ser Gly Phe Gly Gly Phe Gln Ser Ala
                405                 410                 415

ATG GTG CTG TGC GGT CCG GGC TCG CGG GGA AGG TCG GCC GCG TGACGGCCGC   1658
Met Val Leu Cys Gly Pro Gly Ser Arg Gly Arg Ser Ala Ala
            420                 425                 430

CGTGGTGGTG ACCGGTCTCG GCGTCGTCGC CCCCACCGGT CTCGGGGTGC GGGAGCACTG    1718

GTCGAGTACG GTCCGGGGGG CGTCGGCGAT CGGACCGGTC ACCCGGTTCG ACGCCGGCCG    1778

GTACCCCAGC AAACTGGCCG GAGAGGTGCC CGGTTTCGTC CCGGAGGACC ATCTGCCCAG    1838

CCGGCTGATG CCGCAGACGG ACCATATGAC GCGCCTGGCG CTCGTCGCGG CGGACTGGGC    1898

CTTCCAGGAC GCCGCCGTGG ACCCGTCGAA GCTGCCGGAG TACGGCGTCG GCGTGGTCAC    1958

CGCGAGTTCG GCGGGGGGGT TCGAATTCGG CCACCGCGAG CTGCAGAACC TGTGGAGCCT    2018

GGGCCCGCAG TACGTCAGCG CGTATCAGTC GTTCGCATGG TTCTATGCCG TGAACACCGG    2078

TCAGGTGTCC ATCCGGCACG GGCTGCGCGG CCCGGGCGGG GTGCTGGTGA CGGAACAGGC    2138

GGGCGGCCTG GACGCCCTTG GGCAGGCCCG GCGGCAGTTG CGGCGCGGAC TGCCGATGGT    2198

GGTCGCGGGA GCCGTTGACG GCTCGCCCTG CCCCTGGGGC TGGGTGGCGC AGCTCAGCTC    2258
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GGGCGGCCTC AGCACGTCGG ACGACCCGCG CCGGGCCTAT CTGCCGTTCG ACGCCGCAGC    2318

CGGCGGACAC GTGCCGGGAG AGGGCGGCGC CCTGCTCGTC CTGGAGAGCG ACGAGTCGGC    2378

CCGGGCGCGC GGGGTGACGC GGTGGTACGG GCGCATCGAT GGGTACGCCG CCACATTCGA    2438

CCCCCCGCCC GGTTCGGGGC GCCCGCCGAA CCTGCTGCGG GCCGCGCAGG CGGCACTGGA    2498

CGACGCGGAG GTCGGACCCG AGGCGGTCGA CGTGGTGTTC GCGGACGCGT CCGGCACCCC    2558

GGACGAGGAC GCGGCGGAGG CCGACGCGGT GCGGCGCCTG TTCGGACCGT ACGGCGTTCC    2618

GGTGACGGCG CCGAAGACCA TGACCGGCCG CCTCAGCGCG GCCGGCGCGG CCCTCGACGT    2678

GGCGACGGCG CTGCTGGCGC TGCGCGAGGG CGTCGTCCCG CCGACGGTCA ACGTCTCCCG    2738

GCCGCGGCCG GAGTACGAGC TGGACCTGGT GCTCGCCCCC CGGCGCACGC CCCTGGCCAG    2798

GGCCCTGGTG CTCGCGCGGG GCCGGGGCGG GTTCAATGCG GCGATGGTCG TGGCGGGGCC    2858

GCGCGCGGAG ACACGGTGAA GCGGCCCGGC GCAGCCGGAG CCGCGGTAAG AGGCCACGGA    2918

AGAGAGAGGG ATGCGACG GTG AAG CAG CAG CTG ACG ACG GAA CGG CTC ATG       2969
                    Val Lys Gln Gln Leu Thr Thr Glu Arg Leu Met
                      1               5                  10

GAG ATC ATG CGG GAG TGC GCG GGC TAC GGT GAG GAC GTC GAC GCT CTG       3017
Glu Ile Met Arg Glu Cys Ala Gly Tyr Gly Glu Asp Val Asp Ala Leu
            15                  20                  25

GGC GAC ACG GAC GGC GCC GAC TTC GCC GCA CTC GGC TAC GAC TCG CTG       3065
Gly Asp Thr Asp Gly Ala Asp Phe Ala Ala Leu Gly Tyr Asp Ser Leu
        30                  35                  40

GCG CTC CTG GAA ACG GCC GGC CGG CTC GAG CGC GAG TTC GGC ATC CAG       3113
Ala Leu Leu Glu Thr Ala Gly Arg Leu Glu Arg Glu Phe Gly Ile Gln
    45                  50                  55

CTC GGT GAC GAG GTG GTC GCC GAC GCC AGG ACG CCT GCC GAG CTG ACC       3161
Leu Gly Asp Glu Val Val Ala Asp Ala Arg Thr Pro Ala Glu Leu Thr
60                  65                  70                  75

GCC CTG GTC AAC CGG ACG GTG GCC GAG GCG GCC TGACCCGGCC GGCCCACGAG     3214
Ala Leu Val Asn Arg Thr Val Ala Glu Ala Ala
                80                  85

AGCGGGGTGA CGCGTGTGTA CGGCACGGAA CTCACACA                             3252
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 430 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Lys Glu Ser Ile Asn Arg Arg Val Val Ile Thr Gly Ile Gly Ile
 1               5                  10                  15

Val Ala Pro Asp Ala Thr Gly Val Lys Pro Phe Trp Asp Leu Leu Thr
            20                  25                  30

Ala Gly Arg Thr Ala Thr Arg Thr Ile Thr Ala Phe Asp Pro Ser Pro
        35                  40                  45
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Phe Arg Ser Arg Ile Ala Ala Glu Cys Asp Phe Asp Pro Leu Ala Glu
 50                  55                  60

Gly Leu Thr Pro Gln Gln Ile Arg Arg Met Asp Arg Ala Thr Gln Phe
65                  70                  75                  80

Ala Val Val Ser Ala Arg Glu Ser Leu Glu Asp Ser Gly Leu Asp Leu
                85                  90                  95

Gly Ala Leu Asp Ala Ser Arg Thr Gly Val Val Val Gly Ser Ala Val
            100                 105                 110

Gly Cys Thr Thr Ser Leu Glu Glu Glu Tyr Ala Val Val Ser Asp Ser
        115                 120                 125

Gly Arg Asn Trp Leu Val Asp Asp Gly Tyr Ala Val Pro His Leu Phe
    130                 135                 140

Asp Tyr Phe Val Pro Ser Ser Ile Ala Ala Glu Val Ala His Asp Arg
145                 150                 155                 160

Ile Gly Ala Glu Gly Pro Val Ser Leu Val Ser Thr Gly Cys Thr Ser
                165                 170                 175

Gly Leu Asp Ala Val Gly Arg Ala Ala Asp Leu Ile Ala Glu Gly Ala
            180                 185                 190

Ala Asp Val Met Leu Ala Gly Ala Thr Glu Ala Pro Ile Ser Pro Ile
        195                 200                 205

Thr Val Ala Cys Phe Asp Ala Ile Lys Ala Thr Thr Pro Arg Asn Asp
    210                 215                 220

Thr Pro Ala Glu Ala Ser Arg Pro Phe Asp Arg Thr Arg Asn Gly Phe
225                 230                 235                 240

Val Leu Gly Glu Gly Ala Ala Val Phe Val Leu Glu Glu Phe Glu His
                245                 250                 255

Ala Arg Arg Arg Gly Ala Leu Val Tyr Ala Glu Ile Ala Gly Phe Ala
            260                 265                 270

Thr Arg Cys Asn Ala Phe His Met Thr Gly Leu Arg Pro Asp Gly Arg
        275                 280                 285

Glu Met Ala Glu Ala Ile Gly Val Ala Leu Ala Gln Ala Gly Lys Ala
    290                 295                 300

Pro Ala Asp Val Asp Tyr Val Asn Ala His Gly Ser Gly Thr Arg Gln
305                 310                 315                 320

Asn Asp Arg His Glu Thr Ala Ala Phe Lys Arg Ser Leu Gly Asp His
                325                 330                 335

Ala Tyr Arg Val Pro Val Ser Ser Ile Lys Ser Met Ile Gly His Ser
            340                 345                 350

Leu Gly Ala Ile Gly Ser Leu Glu Ile Ala Ala Ser Val Leu Ala Ile
        355                 360                 365

Thr His Asp Val Val Pro Pro Thr Ala Asn Leu His Glu Pro Asp Pro
    370                 375                 380

Glu Cys Asp Leu Asp Tyr Val Pro Leu Arg Ala Arg Ala Cys Pro Val
385                 390                 395                 400

Asp Thr Val Leu Thr Val Gly Ser Gly Phe Gly Gly Phe Gln Ser Ala
                405                 410                 415
```

Met Val Leu Cys Gly Pro Gly Ser Arg Gly Arg Ser Ala Ala
420 425 430

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 86 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Val Lys Gln Gln Leu Thr Thr Glu Arg Leu Met Glu Ile Met Arg Glu
1 5 10 15

Cys Ala Gly Tyr Gly Glu Asp Val Asp Ala Leu Gly Asp Thr Asp Gly
20 25 30

Ala Asp Phe Ala Ala Leu Gly Tyr Asp Ser Leu Ala Leu Leu Glu Thr
35 40 45

Ala Gly Arg Leu Glu Arg Glu Phe Gly Ile Gln Leu Gly Asp Glu Val
50 55 60

Val Ala Asp Ala Arg Thr Pro Ala Glu Leu Thr Ala Leu Val Asn Arg
65 70 75 80

Thr Val Ala Glu Ala Ala
85

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3252 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: *Streptomyces nogalater* ATCC 27451

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1648..2877
(D) OTHER INFORMATION: /note= "ORF2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAATTCGGCC GTACCCCGAC GGCCGATTCC TTACCCTTCC GGAGCGGCTT GCGGATCGCA 60

GGACGAAGTC CTCCCTCTCC CCCCATCGGG CGTCCGCTCT TTGTGACCGG TTCACGAGTC 120

GGGTTCCAGC GCTCCTCGAC TCAGGATCGA CCCCTTCCGC GGTAGCCGCC CCGCAGGAAC 180

CGCAAACCTT CCGCGCCGGT CCCGCCGGGC TTCGCCGCAC CCGTCCATCC GTCATTGAGC 240

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TGATTTCGAG ACAGGACGCG CACTGTCACC ACGAGCCCTG TGCGGTTGAA GTCATCACCT      300

GTCCGCGCAC AGGAACTTCA AGACGATCAA AGCCCCTAGT GAAGGGCATC TTCGACGAAT      360

GAAGGAATCC ATCAACCGTC GCGTGGTCAT CACCGGAATA GGGATCGTCG CGCCCGATGC      420

CACCGGGGTG AAACCGTTCT GGGATCTGCT GACGGCCGGT CGCACCGCGA CCCGGACCAT      480

CACCGCCTTC GATCCCTCTC CGTTCCGTTC CCGCATCGCC GCGGAATGCG ATTTCGACCC      540

GCTTGCCGAA GGGCTGACCC CCCAGCAGAT CCGGCGTATG GACCGGGCCA CGCAGTTCGC      600

GGTCGTCAGC GCCCCGGGAAA GCCTGGAGGA CAGCGGACTC GACCTCGGCG CCCTGGACGC     660

CTCCCGCACC GGCGTGGTCG TCGGCAGCGC GGTCGGCTGC ACCACGAGCC TGGAAGAGGA      720

GTACGCGGTC GTCAGCGACA GCGGCCGGAA CTGGCTGGTC GACGACGGCT ACGCCGTACC      780

GCACCTATTC GACTACTTCG TGCCCAGCTC CATCGCCGCC GAGGTGGCAC ACGACCGCAT      840

CGGCGCGGAG GGCCCCGTCA GCCTCGTGTC GACCGGGTGC ACCTCGGGCC TGGACGCCGT      900

GGGCCGCGCG GCCGACCTGA TCGCCGAGGG AGCGGCGGAT GTGATGCTGG CCGGTGCGAC      960

CGAGGCGCCC ATCTCCCCCA TCACCGTGGC GTGCTTCGAT GCCATCAAGG CGACCACCCC     1020

CCGCAACGAC ACGCCCGCCG AGGCGTCCCG TCCGTTCGAC CGCACCAGGA ACGGGTTCGT     1080

ACTCGGCGAG GGCGCTGCCG TGTTCGTCCT GGAGGAGTTC GAACACGCGC GCCGCCGGGG     1140

CGCGCTCGTG TACGCGGAGA TCGCCGGGTT CGCCACTCGC TGCAACGCCT TCCACATGAC     1200

CGGTCTGCGC CCGGACGGGC GGGAGATGGC GGAGGCCATC GGGGTGGCGC TCGCCCAGGC     1260

GGGCAAGGCG CCGGCTGACG TCGACTACGT CAACGCCCAC GGTTCCGGCA CCCGGCAGAA     1320

TGACCGTCAC GAGACGGCGG CCTTCAAGCG CAGTCTCGGC GACCACGCCT ACCGGGTCCC     1380

GGTCAGCAGC ATCAAATCCA TGATCGGGCA CTCGCTGGGC GCGATCGGCT CCCTGGAGAT     1440

CGCCGCCTCC GTGCTGGCCA TCACACACGA CGTGGTGCCG CCCACCGCCA ATCTGCACGA     1500

GCCGGATCCC GAGTGCGATC TGGACTACGT GCCGCTGCGG GCGCGTGCGT GCCCGGTGGA     1560

CACGGTGCTC ACGGTGGGCA GCGGGTTCGG CGGTTTCCAG AGCGCCATGG TGCTGTGCGG     1620

TCCGGGCTCG CGGGGAAGGT CGGCCGC GTG ACG GCC GCC GTG GTG GTG ACC          1671
                               Val Thr Ala Ala Val Val Val Thr
                                 1               5

GGT CTC GGC GTC GTC GCC CCC ACC GGT CTC GGG GTG CGG GAG CAC TGG       1719
Gly Leu Gly Val Val Ala Pro Thr Gly Leu Gly Val Arg Glu His Trp
     10                  15                  20

TCG AGT ACG GTC CGG GGG GCG TCG GCG ATC GGA CCG GTC ACC CGG TTC       1767
Ser Ser Thr Val Arg Gly Ala Ser Ala Ile Gly Pro Val Thr Arg Phe
 25                  30                  35                  40

GAC GCC GGC CGG TAC CCC AGC AAA CTG GCC GGA GAG GTG CCC GGT TTC       1815
Asp Ala Gly Arg Tyr Pro Ser Lys Leu Ala Gly Glu Val Pro Gly Phe
                 45                  50                  55

GTC CCG GAG GAC CAT CTG CCC AGC CGG CTG ATG CCG CAG ACG GAC CAT       1863
Val Pro Glu Asp His Leu Pro Ser Arg Leu Met Pro Gln Thr Asp His
            60                  65                  70
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ATG ACG CGC CTG GCG CTC GTC GCG GCG GAC TGG GCC TTC CAG GAC GCC      1911
Met Thr Arg Leu Ala Leu Val Ala Ala Asp Trp Ala Phe Gln Asp Ala
         75                  80                  85

GCC GTG GAC CCG TCG AAG CTG CCG GAG TAC GGC GTC GGC GTG GTC ACC      1959
Ala Val Asp Pro Ser Lys Leu Pro Glu Tyr Gly Val Gly Val Val Thr
     90                  95                 100

GCG AGT TCG GCG GGG GGG TTC GAA TTC GGC CAC CGC GAG CTG CAG AAC      2007
Ala Ser Ser Ala Gly Gly Phe Glu Phe Gly His Arg Glu Leu Gln Asn
105                 110                 115                 120

CTG TGG AGC CTG GGC CCG CAG TAC GTC AGC GCG TAT CAG TCG TTC GCA      2055
Leu Trp Ser Leu Gly Pro Gln Tyr Val Ser Ala Tyr Gln Ser Phe Ala
                125                 130                 135

TGG TTC TAT GCC GTG AAC ACC GGT CAG GTG TCC ATC CGG CAC GGG CTG      2103
Trp Phe Tyr Ala Val Asn Thr Gly Gln Val Ser Ile Arg His Gly Leu
            140                 145                 150

CGC GGC CCG GGC GGG GTG CTG GTG ACG GAA CAG GCG GGC GGC CTG GAC      2151
Arg Gly Pro Gly Gly Val Leu Val Thr Glu Gln Ala Gly Gly Leu Asp
        155                 160                 165

GCC CTT GGG CAG GCC CGG CGG CAG TTG CGG CGC GGA CTG CCG ATG GTG      2199
Ala Leu Gly Gln Ala Arg Arg Gln Leu Arg Arg Gly Leu Pro Met Val
    170                 175                 180

GTC GCG GGA GCC GTT GAC GGC TCG CCC TGC CCC TGG GGC TGG GTG GCG      2247
Val Ala Gly Ala Val Asp Gly Ser Pro Cys Pro Trp Gly Trp Val Ala
185                 190                 195                 200

CAG CTC AGC TCG GGC GGC CTC AGC ACG TCG GAC GAC CCG CGC CGG GCC      2295
Gln Leu Ser Ser Gly Gly Leu Ser Thr Ser Asp Asp Pro Arg Arg Ala
                205                 210                 215

TAT CTG CCG TTC GAC GCC GCA GCC GGC GGA CAC GTG CCG GGA GAG GGC      2343
Tyr Leu Pro Phe Asp Ala Ala Ala Gly Gly His Val Pro Gly Glu Gly
            220                 225                 230

GGC GCC CTG CTC GTC CTG GAG AGC GAC GAG TCG GCC CGG GCG CGC GGG      2391
Gly Ala Leu Leu Val Leu Glu Ser Asp Glu Ser Ala Arg Ala Arg Gly
        235                 240                 245

GTG ACG CGG TGG TAC GGG CGC ATC GAT GGG TAC GCC GCC ACA TTC GAC      2439
Val Thr Arg Trp Tyr Gly Arg Ile Asp Gly Tyr Ala Ala Thr Phe Asp
    250                 255                 260

CCC CCG CCC GGT TCG GGG CGC CCG CCG AAC CTG CTG CGG GCC GCG CAG      2487
Pro Pro Pro Gly Ser Gly Arg Pro Pro Asn Leu Leu Arg Ala Ala Gln
265                 270                 275                 280

GCG GCA CTG GAC GAC GCG GAG GTC GGA CCC GAG GCG GTC GAC GTG GTG      2535
Ala Ala Leu Asp Asp Ala Glu Val Gly Pro Glu Ala Val Asp Val Val
                285                 290                 295

TTC GCG GAC GCG TCC GGC ACC CCG GAC GAG GAC GCG GCG GAG GCC GAC      2583
Phe Ala Asp Ala Ser Gly Thr Pro Asp Glu Asp Ala Ala Glu Ala Asp
            300                 305                 310

GCG GTG CGG CGC CTG TTC GGA CCG TAC GGC GTT CCG GTG ACG GCG CCG      2631
Ala Val Arg Arg Leu Phe Gly Pro Tyr Gly Val Pro Val Thr Ala Pro
        315                 320                 325

AAG ACC ATG ACC GGC CGC CTC AGC GCG GGC GGC GCG GCC CTC GAC GTG      2679
Lys Thr Met Thr Gly Arg Leu Ser Ala Gly Gly Ala Ala Leu Asp Val
    330                 335                 340
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GCG ACG GCG CTG CTG GCG CTG CGC GAG GGC GTC GTC CCG CCG ACG GTC      2727
Ala Thr Ala Leu Leu Ala Leu Arg Glu Gly Val Val Pro Pro Thr Val
345                 350                 355                 360

AAC GTC TCC CGG CCG CGG CCG GAG TAC GAG CTG GAC CTG GTG CTC GCC      2775
Asn Val Ser Arg Pro Arg Pro Glu Tyr Glu Leu Asp Leu Val Leu Ala
                365                 370                 375

CCC CGG CGC ACG CCC CTG GCC AGG GCC CTG GTG CTC GCG CGG GGC CGG      2823
Pro Arg Arg Thr Pro Leu Ala Arg Ala Leu Val Leu Ala Arg Gly Arg
            380                 385                 390

GGC GGG TTC AAT GCG GCG ATG GTC GTG GCG GGG CCG CGC GCG GAG ACA      2871
Gly Gly Phe Asn Ala Ala Met Val Val Ala Gly Pro Arg Ala Glu Thr
        395                 400                 405

CGG TGAAGCGGCC CGGCGCAGCC GGAGCCGCGG TAAGAGGCCA CGGAAGAGAG           2924
Arg
    410

AGGGATGCGA CGGTGAAGCA GCAGCTGACG ACGGAACGGC TCATGGAGAT CATGCGGGAG    2984

TGCGCGGGCT ACGGTGAGGA CGTCGACGCT CTGGGCGACA CGGACGGCGC CGACTTCGCC    3044

GCACTCGGCT ACGACTCGCT GGCGCTCCTG GAAACGGCCG GCCGGCTCGA GCGCGAGTTC    3104

GGCATCCAGC TCGGTGACGA GGTGGTCGCC GACGCCAGGA CGCCTGCCGA GCTGACCGCC    3164

CTGGTCAACC GGACGGTGGC CGAGGCGGCC TGACCCGGCC GGCCCACGAG AGCGGGGTGA    3224

CGCGTGTGTA CGGCACGGAA CTCACACA                                       3252
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 409 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Val Thr Ala Ala Val Val Val Thr Gly Leu Gly Val Val Ala Pro Thr
 1               5                  10                  15

Gly Leu Gly Val Arg Glu His Trp Ser Ser Thr Val Arg Gly Ala Ser
            20                  25                  30

Ala Ile Gly Pro Val Thr Arg Phe Asp Ala Gly Arg Tyr Pro Ser Lys
        35                  40                  45

Leu Ala Gly Glu Val Pro Gly Phe Val Pro Glu Asp His Leu Pro Ser
    50                  55                  60

Arg Leu Met Pro Gln Thr Asp His Met Thr Arg Leu Ala Leu Val Ala
65                  70                  75                  80

Ala Asp Trp Ala Phe Gln Asp Ala Ala Val Asp Pro Ser Lys Leu Pro
                85                  90                  95

Glu Tyr Gly Val Gly Val Val Thr Ala Ser Ser Ala Gly Gly Phe Glu
            100                 105                 110

Phe Gly His Arg Glu Leu Gln Asn Leu Trp Ser Leu Gly Pro Gln Tyr
        115                 120                 125
```

Val Ser Ala Tyr Gln Ser Phe Ala Trp Phe Tyr Ala Val Asn Thr Gly
130 135 140

Gln Val Ser Ile Arg His Gly Leu Arg Gly Pro Gly Gly Val Leu Val
145 150 155 160

Thr Glu Gln Ala Gly Gly Leu Asp Ala Leu Gly Gln Ala Arg Arg Gln
165 170 175

Leu Arg Arg Gly Leu Pro Met Val Val Ala Gly Ala Val Asp Gly Ser
180 185 190

Pro Cys Pro Trp Gly Trp Val Ala Gln Leu Ser Ser Gly Gly Leu Ser
195 200 205

Thr Ser Asp Asp Pro Arg Arg Ala Tyr Leu Pro Phe Asp Ala Ala Ala
210 215 220

Gly Gly His Val Pro Gly Glu Gly Gly Ala Leu Leu Val Leu Glu Ser
225 230 235 240

Asp Glu Ser Ala Arg Ala Arg Gly Val Thr Arg Trp Tyr Gly Arg Ile
245 250 255

Asp Gly Tyr Ala Ala Thr Phe Asp Pro Pro Pro Gly Ser Gly Arg Pro
260 265 270

Pro Asn Leu Leu Arg Ala Ala Gln Ala Ala Leu Asp Asp Ala Glu Val
275 280 285

Gly Pro Glu Ala Val Asp Val Val Phe Ala Asp Ala Ser Gly Thr Pro
290 295 300

Asp Glu Asp Ala Ala Glu Ala Asp Ala Val Arg Arg Leu Phe Gly Pro
305 310 315 320

Tyr Gly Val Pro Val Thr Ala Pro Lys Thr Met Thr Gly Arg Leu Ser
325 330 335

Ala Gly Gly Ala Ala Leu Asp Val Ala Thr Ala Leu Leu Ala Leu Arg
340 345 350

Glu Gly Val Val Pro Pro Thr Val Asn Val Ser Arg Pro Arg Pro Glu
355 360 365

Tyr Glu Leu Asp Leu Val Leu Ala Pro Arg Arg Thr Pro Leu Ala Arg
370 375 380

Ala Leu Val Leu Ala Arg Gly Arg Gly Gly Phe Asn Ala Ala Met Val
385 390 395 400

Val Ala Gly Pro Arg Ala Glu Thr Arg
405

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 26, line 25.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM)

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1 b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
15 September 1994	DSM 9436

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☒

In respect of those designations in which a European patent or a patent in Finland or Norway is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or the corresponding information concerning the patent in Finland or Norway or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC and the corresponding regulations in Finland and Norway).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer Niina Aleilouski	Authorized officer

Form PCT/RO/134 (July 1992)

Indications relating to deposited microorganisms
Continuation to C. ADDITIONAL INDICATIONS

DSM 9436

When designating Australia, in accordance with regulation 3.25 of the Patents Regulations (Australia Statutory Rules 1991 No. 71), samples of materials deposited in accordance with the Budapest Treaty in relation to this Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

Claims

1. Isolated and purified DNA-fragment, which is a gene fragment of the anthracycline biosynthetic pathway of the bacterium *Streptomyces nogalater* being included in an *actI*-hybridizing 12 kb *Bgl*II-fragment of *S. nogalater* genome.

2. DNA-fragment according to claim 1, which comprises the nucleotide sequence given in SEQ ID NO:1 or a functional part thereof.

3. Recombinant-DNA-construction, which comprises the DNA-fragment according to claim 1 or 2, included in a plasmid which can be transferred into a *Streptomyces*-bacterium and is copied therein.

4. Recombinant-DNA-construction according to claim 3 which is the plasmid pSY15, the structure of which is given in Fig. 3, and which was deposited in *S. lividans* strain TK24/pSY15 with the deposition number DSM 9436.

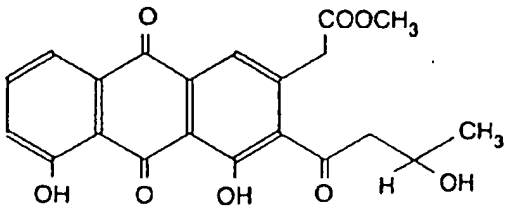
5. Process for the production of anthracyclines and precursors thereof, comprising transferring the DNA-fragment according to claim 1 or 2 into a foreign *Streptomyces* host, cultivating the recombinant strain obtained, and isolating the products formed.

6. Process according to claim 5, wherein the *Streptomyces* host is *S. lividans* or *S. galilaeus*.

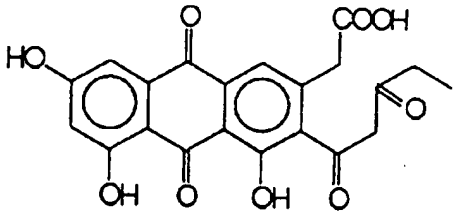
7. Process according to claim 5 for producing auramycinone or glycosides thereof, comprising transferring the DNA-fragment according to claim 2 into *Streptomyces galilaeus* host or a mutant thereof, cultivating the recombinant strain so obtained and isolating auramycinone or a glycoside thereof as formed.

8. Process according to claim 7, wherein the *Streptomyces galilaeus* host is the mutant strain H028 of *S. galilaeus* ATCC 31615.

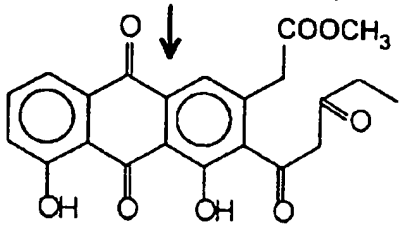
9. An anthracycline precursor which is obtainable according to claim 5 and has the following formula I



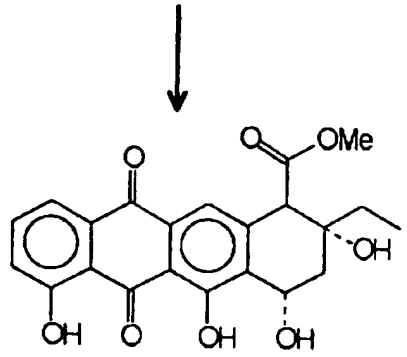
A) (starting unit: propionate)

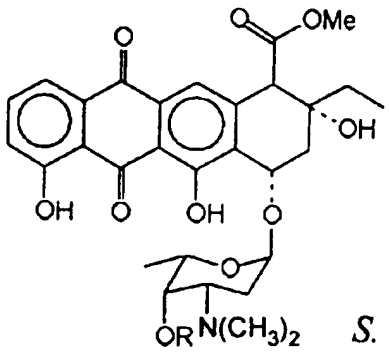


2-OH-aklanone acid (H061)



Methyl ester of aklanone acid (H036)

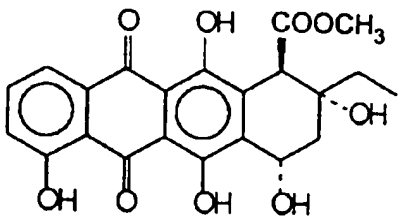


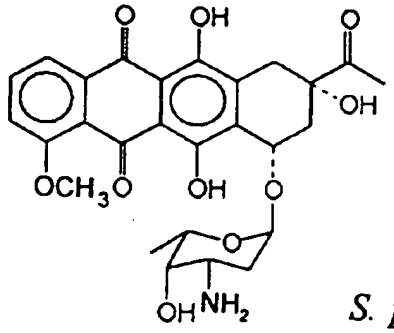


S. *galilaeus*

Aklavinone (H039)

Aclacinomycin





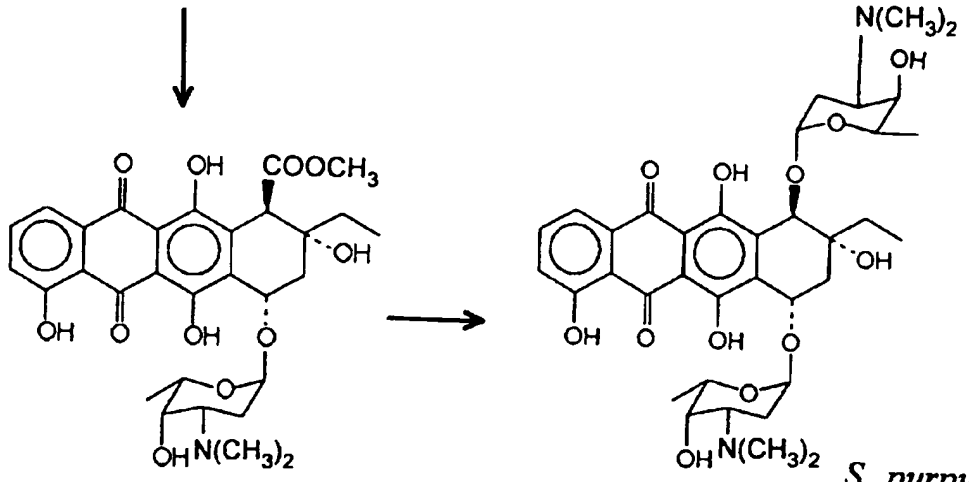
S. *peucetius*

ε-rhodomycinone

Daunorubicin

Fig. 1A/1

ε-rhodomycinone

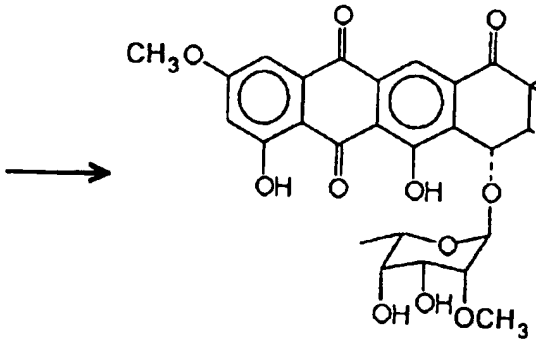


S. purpurascens

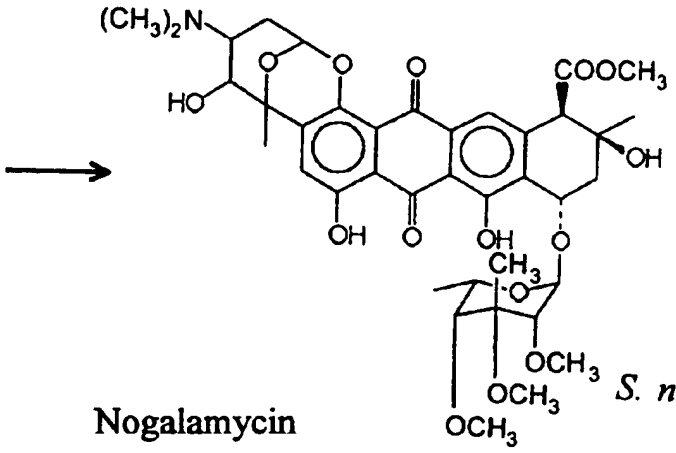
Betaclamycin (H038/EB3) Rhodomycin

Fig. 1A/2

B) (starting unit: acetate)



Steffimycin *S. steffisburgensis*



Nogalamycin *S. nogalater*

Fig. 1B

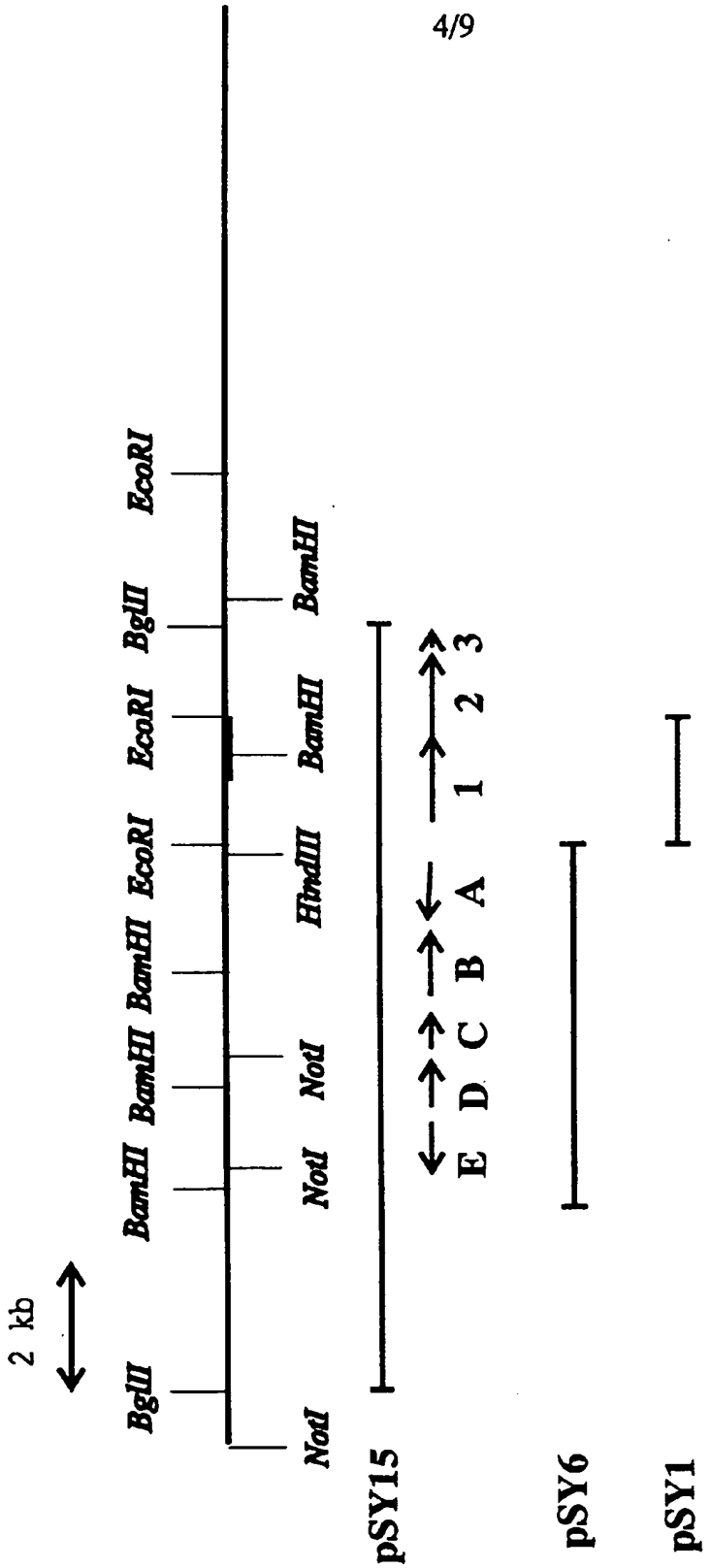

2 kb
BglII
BamHI
BamHI
BamHI
NotI
NotI
NotI
EcoRI
HindIII
BamHI
EcoRI
BglII
BamHI
EcoRI
E D C B A 1 2 3
pSY15
pSY6
pSY1

Fig. 2

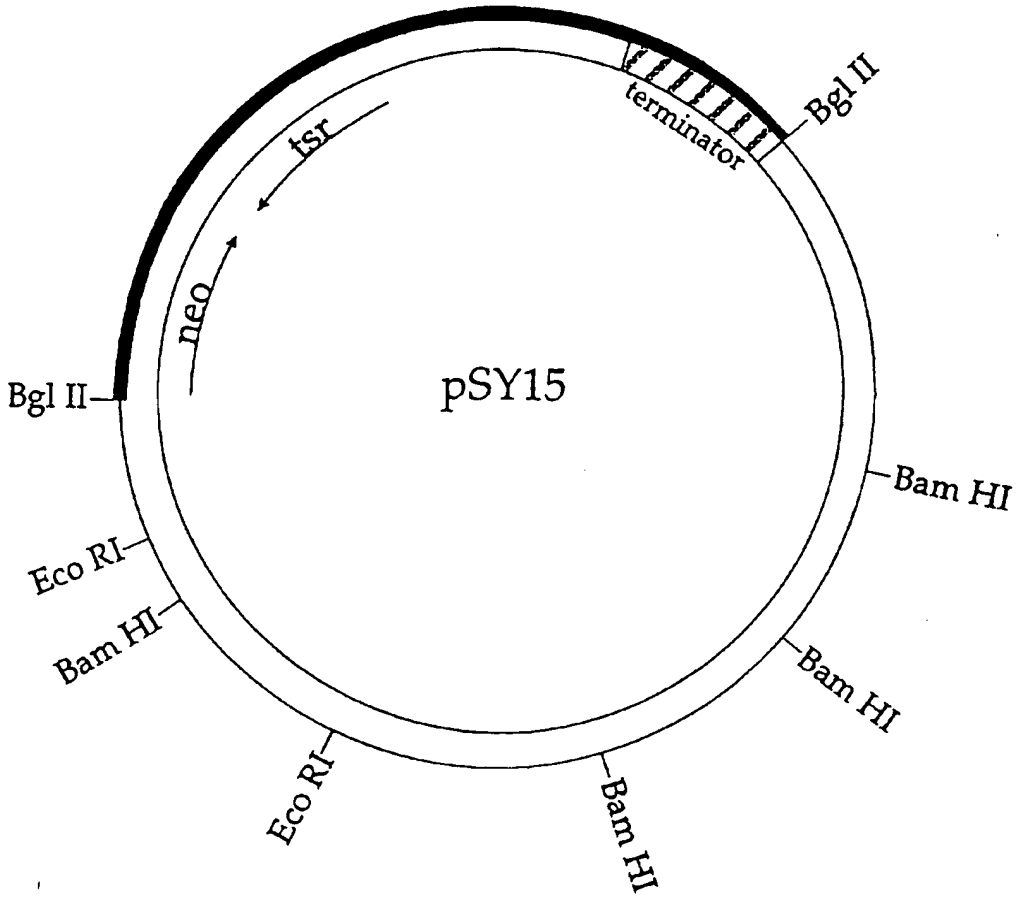

terminator
Bgl II
tsr
neo
pSY15
Bgl II
Bam HI
Eco RI
Bam HI
Bam HI
Eco RI
Bam HI
= pIJ486 (6.2 kb)
= pSY15 insert (12 kb)

Fig. 3

Fig. 4

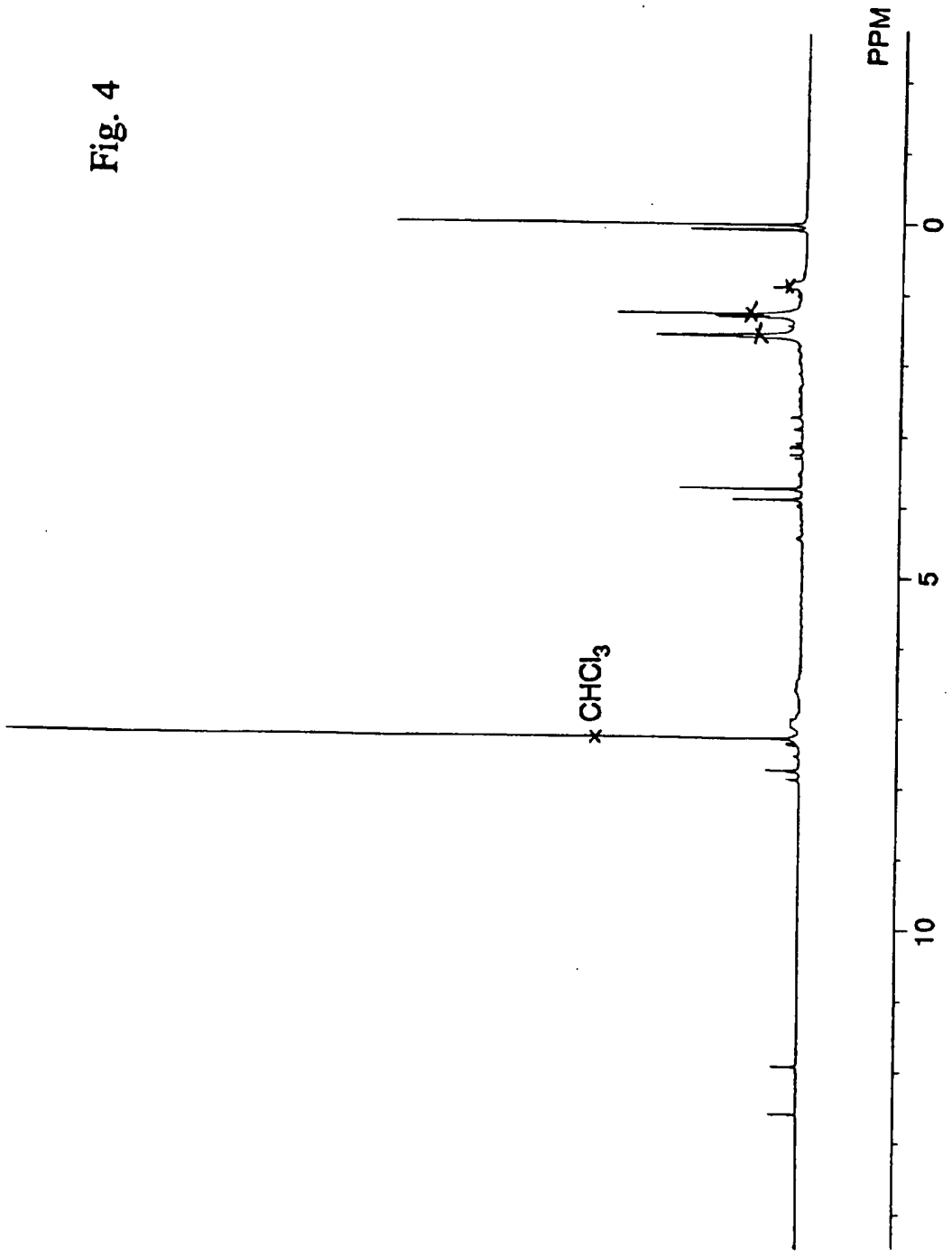


Fig. 5

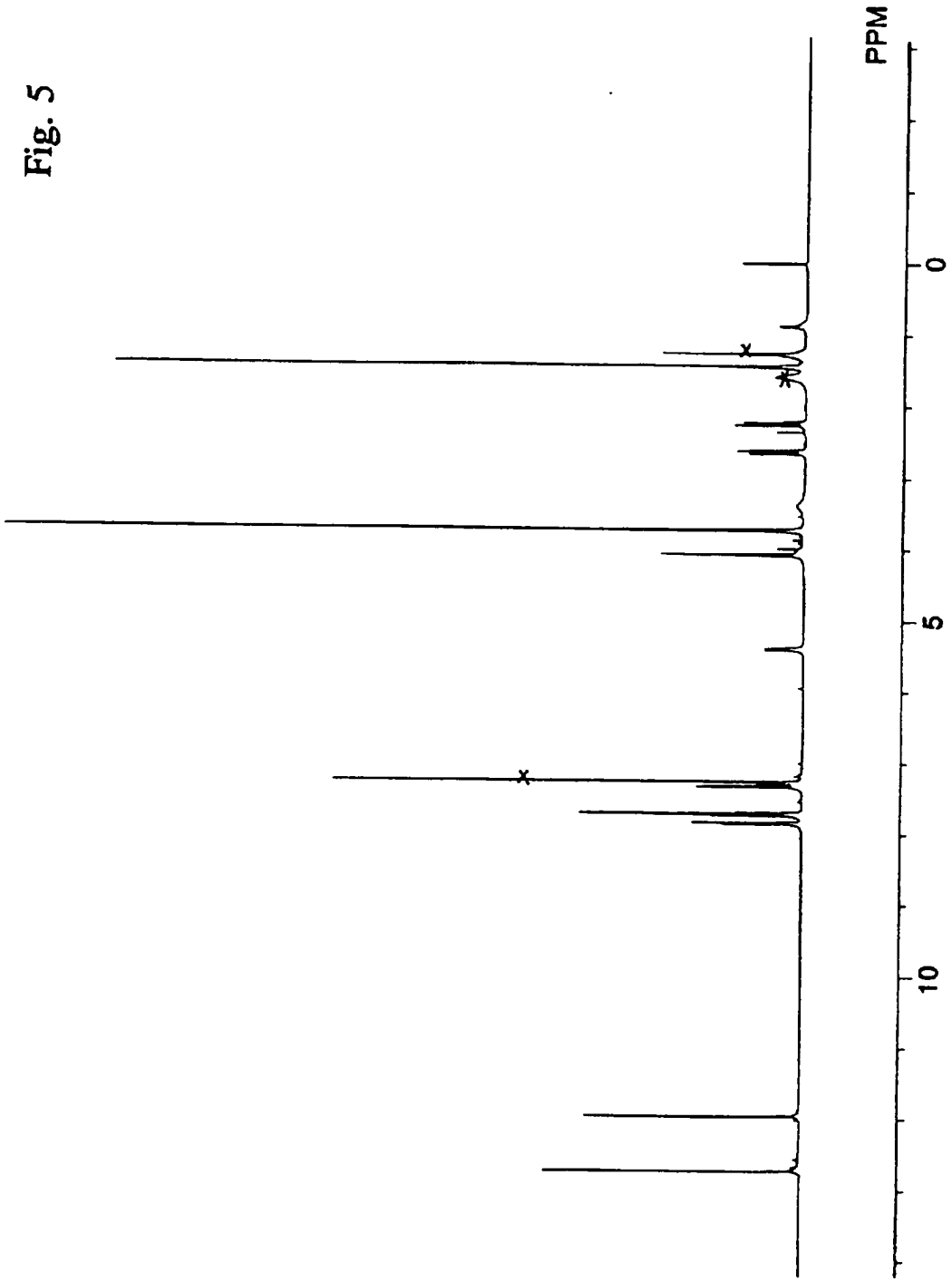


Fig. 6

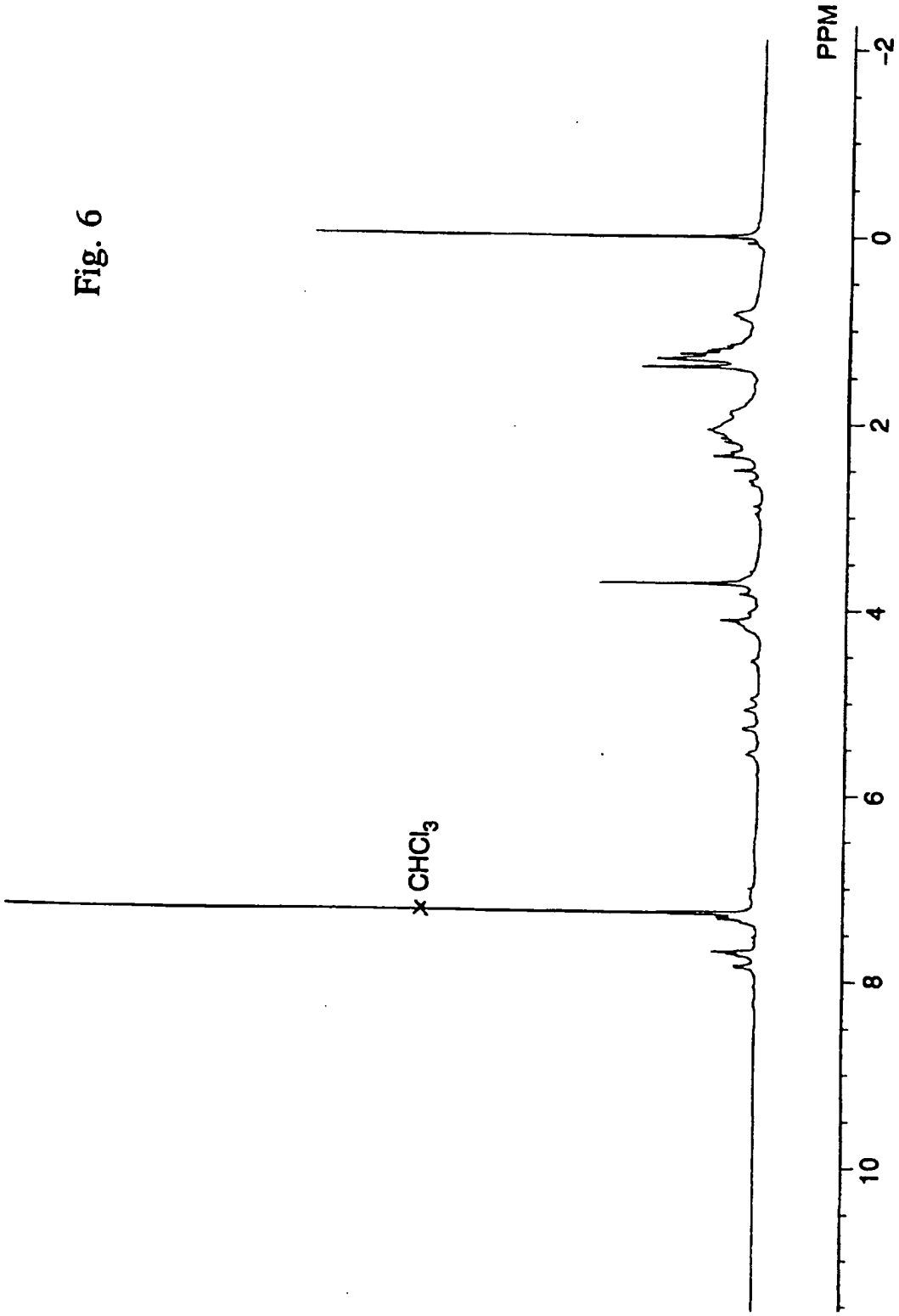
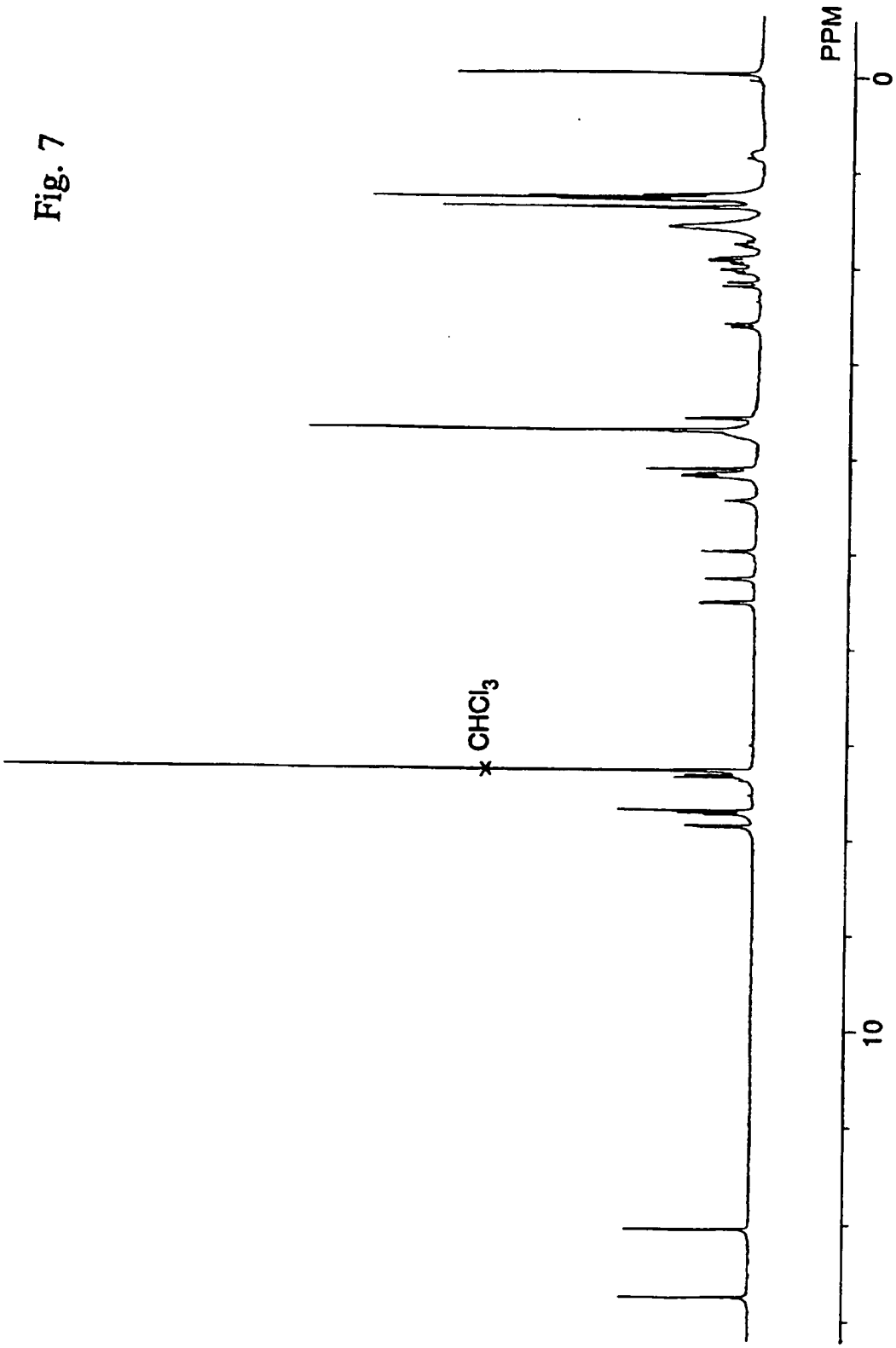


Fig. 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 95/00537

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/36, C12N 15/31, C12P 19/56
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, US PATENT FULLTEXT DATABASES, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proc.Natl.Acad.Sci., Volume 86, May 1989, Kim J. Stutzman-Engwall et al, "Multigene families for anthracycline antibiotic production in Streptomyces peucetius", page 3135 - page 3139, page 3135 left column; page 3136 right column	1
A		2-9
	--	
X	WO 9216629 A1 (LEIRAS OY), 1 October 1992 (01.10.92), page 6, line 6 - line 17; page 8, line 35 - page 9, line 5	1
A	the claims	2-9
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 1996	07 -02- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 95/00537

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBL, Accession No:S52400, Ylihonko et al: "Characterization of the polyketide synthase gene cluster from the nogalamycin producer Streptomyces nogalater", & submitted to the EMBL Data Library, February 1995 -- --------	1-8

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 95/00537

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see extra sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

The wording "... a gene fragment... included in an act I-hybridizing 12 kb Bgl II fragment of S. nogalater genome" of claim 1 is not considered to sufficiently characterize the intended DNA-fragment.
Therefore, claims 1,3,5,6 are not considered to fulfil the requirement of clarity and consiceness according to PCT, Article 6.

INTERNATIONAL SEARCH REPORT

Information on patent family members

05/01/96

International application No.
PCT/FI 95/00537

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9216629	01/10/92	AU-A- 1446192	21/10/92
		FI-B,C- 93860	28/02/95

Form PCT/ISA/210 (patent family annex) (July 1992)